DISSERTATION

EVOLUTIONARY STUDIES IN THE GENUS CUCUMIS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY FENNECHIENA KLOEN DANE ENTITLED EVOLUTIONARY STUDIES IN THE GENUS CUCUMIS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

EVOLUTIONARY STUDIES IN THE GENUS *CUCUMIS*

The evolution of the wild and cultivated *Cucumis* species was studied by the analysis and comparison of (1) their morphological characteristics, (2) their self-compatibility and intra- and interspecific cross-compatibility levels, (3) their basic chromosome number and meiotic chromosome behavior and that of their interspecific hybrids, (4) the variation, inheritance and geographical distribution of their electrophoretically detectable peroxidase isozymes, (5) their glutamate oxaloacetate transaminase isozyme patterns obtained after polyacrylamide gel electrophoresis, and (6) their peroxidase and esterase banding patterns obtained after isoelectric focusing. Immunological studies with antiserum to *C. sativus* peroxidases were used to determine the identity, partial or non identity of the antigenic determinant sites of the *Cucumis* peroxidases.

The genus can be divided into a group of cross-compatible species, distributed over South, East and West Africa, and a group of cross-incompatible species, which includes the two economically important species, *C. melo* and *C. sativus*, and three South African species. Ass species except *C. sativus* (x=7) have a basic chromosome number of x=12. The cross-compatible group of species

contains two allotetraploid, one autotetraploid and one hexaploid species, all of which are perennial, five annual diploid (including C. anguria) and two perennial diploid species. Meiotic chromosome studies of interspecific hybrids indicated that the diploid and polyploid species have one genome in common and that the hexaploid has two genomes in common with one allotetraploid species. Allotetraploidy was not accompanied by an increase in the number of multiporate (4) pollen, but autotetraploidy and hexaploidy was. The annual species have the strongest cross-incompatibility barriers, the polyploid species, with the exception of the autotetraploid, the weakest. Chromosomal rearrangements have exerted an influence on the evolution of the Cucumis species since one interspecific hybrid was heterozygous for a translocation and others had a reduction in bivalent chromosome configurations or pollen fertility.

Most species displayed a homozygous peroxidase banding pattern, characterized by peroxidases at one to three genetic loci (Px_1, Px_2 and Px_3). Genetic polymorphism was observed at the Px_2 locus in four species and at the Px_3 locus in two species. The variant peroxidase alleles were codominantly inherited. Geographic variation in the Px_2 alleles of C. melo, each represented by a cluster of three bands, could not be correlated with variation in morphological characters and may have been the result of inbreeding or differential selection pressures. An increase in ploidy level had little or no effect on the activity or electrophoretic mobility of the isozymes.

South Africa can be considered as the primary gene center of the genus. Only species from the successful cross-compatible group moved northward to establish secondary gene centers. The phylogenetic relationships between the wild species is discussed. C. melo was found to be closer related to three annual South African species (C. africanus, C. leptodermis and C. myriocarpus) than to other Cucumis species. C. sativus was closer related to one annual (C. metuliferus) and one perennial (C. asper) species than to other Cucumis species.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF APPENDIX TABLES

LIST OF FIGURES

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TO THE MEMORY OF

Dr. D. W. Denna

INTRODUCTION

Systematic studies in the genus Cucumis have been based primarily on variations in morphological characters, which are not only controlled by many genes with small individual effects, but may be greatly influenced by the environment, and have resulted in frequent misclassifications of Cucumis plant material. Mutations affecting morphogenetic processes have been used in population genetic studies but failed to characterize species or assist in tracing phylogenies.

With the discovery of enzyme multiplicity and the refinement of biochemical techniques came the possibility of the characterization of the genetic variation within and between species. Isozymic variants usually occur spontaneously, represent single gene products and are generally codominant, making it possible to equate phenotype with genotype. One enzyme which is highly polymorphic and ubiquitous in plant tissues is peroxidase. Its high turnover value, thermostability and monomeric structure make the enzyme uniquely suited as genetic marker.

Speciation can be caused by geographic isolation of populations resulting in the development of reproductive isolation barriers and the selection for ecological divergence. It can occur gradually by changes in the structure or regulation of genes or instantaneously by structural chromosome changes or chromosome doubling. Since

polyploidy has exerted a major influence on the evolution of plant species, mitotic and meiotic chromosome studies of the wild and cultivated Cucumis species are extremely useful.

An understanding of the evolutionary behavior of the wild and cultivated Cucumis species may eventually result in an exchange of genes between the species and open a potential area of variability for exploitation by plant breeders attempting to improve the cultivated Cucumis species.

LITERATURE REVIEW

The Genus Cucumis

The genus Cucumis contains three cultivated species, C. melo, the muskmelon or cantaloupe, C. sativus, the cucumber, and C. anguria, the West Indian gherkin, widely known as vegetables and many wild species, grown as ornamentals or known for their bitter medicinal or poisonous compounds.

Phytogeographic and host-parasite relationships point to North East Africa, Arabia and the eastern Mediterranean area as the primary gene center of the genus Cucumis. From this area species radiated in several directions, establishing a secondary gene center of grassland species in South Africa, and a tertiary gene center in India, with the cucumber and its wild relatives as main representatives (Leppik, 1966). The West Indian gherkin known in cultivated or semi-wild state in the West Indies, is considered conspecific to a wild Cucumis species, C. longipes, indigenous to Africa. Meeuse (1958) found slight morphological differences between his collections of the two species, obtained highly fertile F_1 and F_2 progenies upon cross-pollination, and concluded that C. anguria is nothing more than a cultigen of C. longipes, introduced into America by slave trade over 300 years ago. The wild form was retained as variety longipes, the

cultivated as variety anguria of C. anguria. Deakin et al. (1971) supported these conclusions because of successful hybridization results between C. anguria var. anguria (PI 196477) and C. anguria var. longipes (PI 282442), and Brown et al. (1969) because of the identical leaf flavonoid patterns of their collections of C. anguria and C. longipes.

The origin of the muskmelon remains unresolved. This highly polymorphic and large species is found wild in Africa, probably its primary gene center (de Candolle, 1882; Whitaker and Davis, 1962), and in India, Afghanistan, Turkey and Iran, where well-developed secondary gene centers occur, although Filov et al. (1973) consider India as its area of origin. Genes for powdery mildew, downy mildew and Macrosporium resistance have all occurred in collections from India and Turkey, while cantaloupe mosaic tolerance and/or resistance was found in material from China and Korea (Whitaker and Bohn, 1954; Corley, 1966). Whitaker and Davis (1962) consider the introduction of Cucumis melo into Asia of relatively late occurrence because of the lack of a Sanskrit name for the muskmelon, while Filov et al. (1973) based their conclusion about the area of origin of this species on linguistic data.

Most species are found in arid and semi-arid regions, in deserts, steppes and savannah forests, while few prefer humid conditions and deep shade. One species, C. humifructus, is the only geocarpic member of the Cucurbitaceae. It is found almost

exclusively near aardvark holes because of its dissemination dependence on those animals. Although the cultivated Cucumis species have an annual growth habit, many of the wild species are perennial herbs. Their usual mode of sex expression is monoecy or less often dioecy, which makes the plants dependent on pollinating insects for survival (Meeuse, 1962).

The first comprehensive monographic treatment of the entire genus was presented by Cogniaux and Harms (1924), who distinguished 37 species based on minute morphological differences between herbarium specimens. The number could later be reduced by taxonomists studying plants in their natural habitats. Meeuse (1962) indentified 17 Cucumis species in South Africa, Jeffrey (1967) 13 in tropical East Africa. Some discrepancies in their species classifications are apparent, in that C. prophetarum (Jeffrey, 1967) appears to be similar to C. zeyheri (Meeuse, 1962). The presence of C. melo and C. sativus in the genus has been questioned. Pangalo (1950) proposed to transfer C. melo to a separate genus Melo Adans.

The ancestry of the cultivated Cucumis species has not yet been established. Interspecific hybridization studies of the cultivated species failed to secure hybrids at the tetraploid as well as at the diploid level (Whitaker, 1933; Batra, 1953; Smith and Venkat Ram, 1954; Deakin et al., 1971). A successful cross between C. melo and C. pubescens (Shanmugasundaram et al., 1964), and C. melo and C. metuliferus (Norton, 1969) was recorded, but appeared to be

intra-specific rather than interspecific (Deakin et al., 1971; Greenleaf, 1974). Interspecific hybridization studies of wild Cucumis species indicated that several spiny fruited species:

C. dipsaceus (PI 193498)	C. africanus (PI 282440)
C. ficifolius (PI 196844)	C. heptadactylus (PI 282446)
C. leptodermis (PI 282447)	C. myriocarpus (PI 282449)
C. prophetarum (PI 292396)	C. zeyheri (PI 282450)

were relatively cross-fertile with and consequently related to C. anguria. The other species were cross-sterile with all other species and divided into three groups based on morphology (Deakin et al., 1971). Cucumis metuliferus with its ring-mottled orange fruits with fleshy spines (so-called "horned cucumber") was placed in a separate group like C. sativus and its wild form, C. hardwickii. Three South African hairy fruited species, C. humifructus, C. dinteri and C. sagittatus were considered more closely related to each other and C. melo than to any of the other species (Deakin et al., 1971). A successful cross between C. anguria and C. humifructus was reported however, suggesting that C. humifructus may belong to the cross-compatible C. anguria group (Andrus and Fassuliotis, 1965).

Chromatographic flavonoid patterns of leaf extracts of 21 Cucumis species failed to conform with groupings based on morphology, but indicated species specific patterns. C. melo was found to have many flavonoids in common with:

C. africanus
C. heptadactylus
C. hookeri
C. leptodermis
C. myriocarpus

and C. anguria's flavonoid pattern showed many similarities with that of:

C. dipsaceus
C. dinteri
C. ficifolius
C. membranifolius
C. prophetarum
C. pustulatus (PI 280231)
C. zeyheri

Cucumis metuliferus, C. humifructus and C. hirsutus were clearly different in chromatographic pattern from each other and the other species. The flavonoid pattern of C. sativus was similar to that of C. hardwickii and C. trigonus (Brown et al., 1969).

Unsuccessful hybridization results between the cucumber and all other Cucumis species can be explained by a difference in basic chromosome number. While the other cultivated and wild African species have a basic chromosome number of x=12 (Table 1), C. sativus and its relatives have 2n=14 chromosomes (Kozuchov, 1930; Whitaker, 1930; Shimotsuma, 1965; Singh and Roy, 1974). Meiotic division in the diploid species of Cucumis was found to be normal, with the formation of 7 II in pollen mother cells of C. sativus, and 12 II in pollen mother cells of the other species (Shimotsuma, 1965).

Since the C. sativus chromosomes, ranging from 2-4.5 μ (Trivedi and Roy, 1970; Turkov et al., 1974) are longer than the C. melo chromosomes ranging from 1.2-2.5 μ (Trivedi and Roy, 1970), and

Table 1. Chromosome number of wild Cucumis species.

Species	Chromosome Number	Literature Reference(s)
C. africanus	2n=24	Shimotsuma (1965)
C. angolensis	2n=24	Shimotsuma (1965), van Elden (1966)
C. anguria	2n=24	Whitaker (1930), Kozuchov (1930), Shimotsuma (1965), van Elden (1966), Singh and Roy (1974)
C. asper	2n=24	Shimotsuma (1965), van Elden (1966)
C. dinteri	2n=24	Shimotsuma (1965), van Elden (1966)
C. dipsaceus	2n=24	Kozuchov (1930), Singh and Roy (1974)
C. ficifolius	2n=48	Shimotsuma (1965), van Elden (1966)
C. figarei	2n=24	Shimotsuma (1965), van Elden (1966)
C. hardwickii	2n=14	Shimotsuma (1965), Singh and Roy (1974)
C. heptadactylus	2n=48	Shimotsuma (1965), van Elden (1966)
C. leptodermis	2n=24	Shimotsuma (1965)
C. metuliferus	2n=24	Kozuchov (1930), Shimotsuma (1965), van Elden (1966)
C. myriocarpus	2n=24	Kozuchov (1930), Whitaker (1930), Shimotsuma (1965), van Elden (1966)
C. prophetarum	2n=24	Kozuchov (1930), Shimotsuma (1965), Trivedi and Roy (1970)
C. zeyheri	2n=24	Shimotsuma (1965), van Elden (1966)

have more secondary constrictions, Levitsky's theory of chromosome fragmentation has been proposed to explain the difference in basic chromosome number (Whitaker, 1930; Kozuchov, 1930; Bhaduri and Bose, 1947). According to this view, the chromosome breaks at the secondary constriction region and the newly formed acentric fragment develops a new centromere, thereby stabilizing itself. Trivedi and Roy (1970) and Singh and Roy (1974), however, suggested that the origin of the basic number of x=7 from 12 may be considered a more likely process since it does not involve the formation of a new centromere. It is thought to occur by tranposition of a small chromosome with a subterminal centromere to another chromosome followed by the degeneration of the centromere into a secondary constriction. Such a process may operate by unequal translocation and loss of an inert heterochromatic centromere.

Tetraploidy has been reported in natural populations of C. melo and C. sativus (Shifriss, 1942, 1950; Lozanov, 1972) at a frequency of 1/5000 to 1/10000 in C. sativus varieties (Shifriss, 1950). The spontaneous formation of autotetraploids may have been associated with the occurrence of polysomaty. Kozuchov (1928) found that exposure of C. sativus seedlings to high or low temperatures resulted in the formation of tetraploid cells, while Ervin (1939) found polysomaty not merely occasionally as the result of temperature fluctuations but of regular occurrence in the periblem of root tips of C. melo varieties. The formation of polysomatic cells was thought to be

caused by a chromosome doubling of resting nuclei, since no spindle abnormality or failure of chromosome separation at metaphase occurred (Ervin, 1941). Tetraploidy in cucumbers, naturally occurring or artificially induced with colchicine, resulted in stunted plants with large dark green strongly serrated leaves, a vigorous growth habit, shorter and thicker but significantly less fruits and reduced fertility (Shifriss, 1950; Grimbly, 1973). Their fertility was reduced because of meiotic irregularities and a higher sensitivity of the 2x pollen to minor temperature fluctuations (Shifriss, 1950). Their chance of survival under present environmental cultivation conditions appeared very small.

Although polyploidy is considered of rare occurrence in the genus Cucumis (Kozuchov, 1930; Whitaker and Davis, 1962), tetraploidy was reported in C. ficifolius, described as a perennial monoecious herb widely distributed in South and East Africa and in C. heptadactylus, a perennial dioecious species of South Africa (Shimotsuma, 1965). A common criterion used in distinguishing auto- from allopolyploids is the frequency with which the chromosomes associate at meiosis in quadrivalents and trivalents instead of the usual bivalent pairing. The homologous chromosomes of an autoploid do not always form multivalents at meiosis, however, since this is dependent on the chiasma frequency of the chromosomes, which in turn is dependent on the chromosome length and is partly genetically controlled. Also, the amount of chromosomal differentiation needed to build a hybrid

sterility barrier between species may be far less than that required to prevent chromosomes from pairing in interspecific hybrids. Although alloploids usually exhibit preferential pairing of homologous chromosomes, multivalent configurations can be formed by pairing of homologous and partly homologous or so-called homoeologous chromosomes (Stebbins, 1971). The tetraploid C. ficifolius showed 24 II or 23 II + 2 I at metaphase I, and C. heptadactylus showed various combinations of hexavalent, quadrivalent, trivalent, bivalent and univalent chromosome configurations at metaphase I, but normal 24-24 anaphase chromosome distributions (Shimotsuma, 1965).

A kind of structural chromosome rearrangement with pronounced effects on gene linkage relationships and evolution is a segmental interchange or reciprocal translocation, which is the result of a breakage and exchange of non-homologous chromosomes. Translocation heterozygotes can be detected cytologically by the presence of multivalent chromosome associations at metaphase I. Abnormal segregation of whole chromosomes or unbalanced combinations of chromosomal segments to the gametes can result in semi-sterility or 50% pollen abortion (Burnham, 1962). Cytogenetic studies of Cucurbita (Weiling, 1959) and Citrullus (Shimotsuma, 1963) species indicated the presence of quadrivalents and trivalents in some interspecific hybrids suggesting that chromosomal rearrangements may have played an important role in the evolution of species in the Cucurbitaceae.

Polyploids can arise from polysomatic cells or unreduced gametes, often visible as larger pollen grains (Burnham, 1962). Funke (1956) found the number of germ pores per pollen grain to be an effective selection technique for tetraploid plants. Diploid Solanum, Anthirrhinum, Trifolium, Nicotiana, and Brassica species had 3-porate pollen, while their natural and artificial tetraploids showed 3-6 pores per pollen grain. Maurizio (1956) reported similar results in other plant species. The phenomenon was more pronounced in auto- than alloploids (Funke, 1956). C. melo pollen are tricolporate and structurally similar to C. sativus pollen but smaller. An increase in ploidy level was found to be associated with an increase in pollen grain size and sterility (Wanjari and Phadnis, 1973).

The relationship between mode of sex expression and fruit shape has been studied in C. melo varieties. Although C. sativus varieties are monoecious, C. melo varieties are monoecious or andromonoecious, containing staminate and pistillate or staminate and perfect flowers respectively. C. melo fruits derived from perfect flowers are spherical in shape (Wall, 1967; Kubicki, 1969). The inheritance of fruit shape was studied and found to be conditioned by two codominant alleles at one locus with round fruit codominant to elongate and oblong as the intermediate fruit form (Bains and Kang, 1963). Although Kubicki (1969) reported that the perfect flowers usually fail to envelope the stigmata and can be easily pollinated by insects, Malinina (1972) examining 500 forms of C. melo found that 63% of the plants

contained stigmata which were completely covered with stamen and consequently tended to breed true. Filov et al. (1971) noticed self-pollination in 70% of the melon varieties. Andromonoecious cucumbers are not cultivated because of ovary underdevelopment and altered ovary and fruit shapes (Kubicki, 1969).

The perfect flower condition of C. melo and C. sativus plants is considered the most primitive form from which the pistillate flowers were derived by a single dominant gene mutation (Kubicki, 1969). Hermaphroditism prevails in 90% of the plant species, with only 7% monoecious and 3% dioecious species. The dioecious species do occur in 75% of the plant families, however, and the vestigial organs of opposite sex found in monosexual flowers suggest them to have originated from bisexual flowers (Westergaard, 1958; Kubicki, 1969). This indicates that monoecy and dioecy in the genus Cucumis must have arisen relatively recently with dioecy probably derived from monoecy (Kubicki, 1969).

Isozymes, Speciation and Evolution

The process of evolution consists of a gradual accumulation of small changes in physiology, morphogenesis and behavior of the organism. Mutation and gene recombination generate the genetic variability underlying the evolutionary process. Determination of the amount and patterns of genetic variation in natural and

experimental populations has been a major problem in population and evolutionary genetics.

The classical theory asserts that a species is essentially homozygous for "wild-type" alleles and that natural selection will act to fix these optional alleles and remove deleterious mutations. A fundamental reason for maintaining this view is related to the problem of genetic load in that too much variability leads to excessive genetic loads (Kimura and Ohta, 1971).

According to the balance theory, a population contains a large amount of potentially adaptive genetic variation with the heterozygosity being maintained by various forms of balancing selection (Lewontin, 1974). Speciation must await the occurrence of new adaptive mutations, according to the classical viewpoint, while the balance hypothesis assumes that genetic variation is always present and that it awaits biogeographic and ecological events.

Evolutionary significant genetic variation was estimated by studying morphological characters, which are not only controlled by many genes with small individual effects but may also be greatly influenced by environmental fluctuations, or some rare recessive mutants, which yield morphological changes but are unlikely to form a representative sample of gene effects (Mayr, 1970). Natural selection experiments remain the strongest evidence of widespread genetic variation for genes of adaptive significant characters (Lewontin, 1974). With the introduction of isozyme techniques to the study of

natural populations came the possibility of the characterization of genetic variation.

The term isozyme, first introduced by Markert and Møller (1959), refers to multiple molecular forms of an enzyme with similar or identical substrate specificity occurring in the same organism. Enzymes with broad substrate specificity were temporarily excluded from the definition but recent investigations of esterases and peroxidases showed their isozymic nature (Scandalios, 1974). The principal kinds of molecular multiplicity known to generate isozymes are:

1. conformational changes generated by permutations of polymer subunits or alternate tertiary conformations;
2. secondary modification of the polypeptide by a variety of chemical and physical means, i.e. binding of a single polypeptide to a varying number of coenzyme molecules or other prosthetic groups, by conjugation or deletion of molecules with reactive groups of amino acid residues of the polypeptide chain;
3. mutation, and gene duplication with subsequent divergence by mutation resulting in molecules with differences in primary structure coded by allelic or nonallelic genes respectively.

Polymeric enzymes, composed of more than one polypeptide chain, may form polymers of different size if the state of aggregation of the enzyme is influenced by the presence of small molecules or assemble

to form homo or heteropolymers with subunits of identical or different primary structure respectively (Whitt, 1967; Markert and Whitt, 1968).

The occurrence of enzyme multiplicity is widespread among plants and animals, indicating that isozymes are the rule rather than the exception as originally thought. Isozymes offer important advantages over conventional characters as genetic markers. Isozymic variants frequently occur spontaneously and seldom produce deleterious effects. Variant alleles represent single gene products and are generally codominant, making it possible to equate phenotype with genotype and identify heterozygotes as well as homozygotes in single individuals (Scandalios, 1969, 1974, 1975; Lewontin, 1974).

Large amounts of electrophoretically detectable genetic variation have been discovered in natural populations of many organisms. Sexually reproducing animal species were found to be polymorphic for 30-40% of their genes with individuals within the species heterozygous for about 10% of their loci. Approximately 31-54% of the loci examined in Avena species were polymorphic (Allard et al., 1971), and genetic variation in haploid E. coli was found to be not very different from that observed in many diploid species (Milkman, 1973). Since electrophoresis detects only a small fraction of allelic substitutions at a locus, it was concluded that many loci are polymorphic and heterozygosity is high (Lewontin, 1974). The question remains whether the isozymes confer different fitnesses on the individuals in which they are functioning or are physiologically irrelevant.

The neoclassical, neo-Darwinian, isoallelic or neutralist theory assumes that the majority of molecular mutations participating in evolution are neutral or indifferent to the action of natural selection. Their fate is controlled by genetic drift due to random sampling of gametes in reproduction. The theory is a renewed version of the classical theory since it assumes that a large fraction of newly arising mutations are deleterious and eliminated by the process of natural selection. The rate of evolutionary change is believed to be independent of environmental fluctuations and population size (Kimura and Ohta, 1971; King and Jukes, 1969).

The alternative or "selection" hypothesis asserts that the polymorphism of populations is a consequence of balancing forms of natural selection, especially heterosis. Heterozygote excesses have been observed at a number of polymorphic loci (Marshall and Allard, 1970; Powell, 1975). Hybrid catalase and alcoholdehydrogenase isozymes of maize generated by allelic or non-allelic interactions showed improved physico-chemical characters over the parental molecules (Scandalios et al., 1972; Felder and Scandalios, 1973).

The neutralist theory predicts that patterns of allelic frequencies should gradually diverge in separate populations and genetically isolated populations should have different sets of allelic frequencies. Also, when geographic variation in allelic frequencies is found, it should not be correlated with environmental factors. Contrary to the prediction, widely separated Drosophila populations were found to

have similar allelic frequencies and showed no geographic differentiation, which could not be explained by migration or gene flow between the populations or by different mutation rates of adaptively equivalent alleles (Lewontin, 1974; Ayala, 1974). Studies with Peromyscus polionotus, the beach mouse, revealed less heterozygosity but more heterogeneity in peninsular geographically isolated populations of Florida than populations in the mainland of the state, pointing to isolation and random fluctuation in gene frequencies (Selander et al., 1971; Lewontin, 1974). Marginal Drosophila populations appear to be as heterozygous as central ones despite great differences in chromosomal polymorphism and despite association of alleles with inversions (Lewontin, 1974). This has been related to the temporal instability of the marginal environments. A correlation of allelic composition and climate was detected in Avena barbata (Allard et al., 1972) but thought to be caused by a linkage effect with unknown alleles under selection pressure (Lewontin, 1974).

Increases in environmental heterogeneity have been correlated with increases in enzyme multiplicity (Bryant, 1974). Powell (1971) showed that the degree of isozyme polymorphism maintained in experimental populations of Drosophila willistoni was directly proportional to the degree of environmental diversity experienced by the populations. Allelic frequencies of serum esterases of fresh water fish Catastomus clarkii were found to vary with latitude because of markedly different temperature regimes of the esterase isozymes

(Koehn, 1969). Somero (1975), however, found that fishes in stable and highly variable temperature habitats did not differ in amount of enzyme polymorphism.

The degree of enzyme multiplicity has also been investigated in relation to the metabolic function of the enzyme. Loci coding for enzymes of animal and insect species which act on substrates originating external to the organism were found to have the highest degree of variation followed by regulatory enzymes followed by non-regulatory enzymes acting on substrates produced internally (Johnson, 1974). A correlation between enzyme multiplicity of metabolically related enzymes was found in 22 plant genera (Sing and Brewer, 1971). Such a regular pattern is highly unlikely if variation is adaptively insignificant.

The demonstration of ontogenetic, subcellular, tissue and metabolic specialization of isozymes lends strong support to their biological significance. Furthermore, isozymic forms are known to have somewhat different kinetic properties, implying that isozymes have been tailored by evolutionary pressures to fit the requirements of the cells metabolic machinery (Markert, 1975).

Electrophoresis, however, does not detect all mutations but only those that cause differences in the net electric charge of the protein molecule, which is around 22% of all possible amino acid substitutions (so called type III mutations). Approximately 32% of the nucleotide base changes do not result in changes in amino acids (type

I mutation) and 46% of nucleotide base changes do result in changes in amino acids but do not change the net electric charge of the protein (type II mutation) (Powell, 1975). A gene coding for a protein molecule contains covarion sites (concomitantly variable codons) which are free to vary and still produce functionally acceptable proteins. Not only are mutations restricted to these sites, but chemically similar amino acids are usually substituted by these sites (Clarke, 1970) which is indicative of selective restraints (Powell, 1975).

Little is known about the adaptive nature of much of the potential variation and evolutionary important nucleotide base changes below the resolving power of the electrophoresis technique. Evolution is probably controlled by both stochastic and deterministic processes (Powell, 1975).

Speciation is thought (Mayr, 1970; Stebbins, 1971; Lewontin, 1974) to be caused by geographic isolation of populations. It restricts the amount of gene exchange between populations and results in ecological divergence and the development of a certain degree of reproductive isolation. The second stage of speciation occurs if isolated popluations come into contact. It is associated with a reinforcement of the reproductive isolation barriers and selection for ecological divergence. The continuous evolution and divergence of species independent of its sibling species is considered the third stage of speciation (Mayr, 1970; Lewontin, 1974). The theory implies that the species are the evolutionary units and that these units are held

together by gene flow. Ehrlich and Raven (1969), however, showed that gene flow is much more restricted than originally thought and that selection is the primary cohesive and disruptive force in evolution. Populations will differentiate if they are under different selection pressures. The local interbreeding population is considered as the evolutionary unit (Ehrlich and Raven, 1969).

Most of the analyses of genetic differences between species have concentrated on the morphological characters used to differentiate them and their isolation mechanism, known to be controlled by a large number of genetic factors with small effects (Mayr, 1970). A study of proteins in ten Drosophila species of the virilis group revealed a direct correlation between protein similarity and cytological or morphological similarity (Hubby and Throckmorton, 1965, 1968). Each species was found to contain approximately 14% "unique" proteins and species divergence was thought to involve 20-30% of the gene pool. Sibling species had 50% of their loci in common, non-sibling species 18%. Allelic frequency patterns of two closely related, cytologically similar Drosophila species showed very little differentiation in gene frequencies (Prakash et al., 1969). Ayala's (1974) studies also indicated lack of allele fixation in a species but showed distinct species similarities and differences. It suggests that only a small portion of the genome may be involved in the formation of sibling species (Gottlieb, 1972).

Ten herbaceous Datura species had unique leaf peroxidase isozyme patterns but their divergence based on cross-compatibility studies could not be correlated with the isozyme data. Three cross-compatible species were quite different in isozyme pattern while one species known to be cross-incompatible with all other Datura species and distinctly different in morphology had many bands in common with three other species (Conklin and Smith, 1971). Agreement between phylogenetic relationships based on conventional methods of systematics and isozyme banding pattern was observed for Nicotiana (Smith et al., 1970), Aegilops (Waines, 1969), Lupinus (Scogin, 1973), and many other species (Peirce and Brewbaker, 1973), but intraspecific variation exceeded interspecific variation in Gossypium species (Cherry et al., 1970) indicating that the preponderance of genetic differences between closely related species is latent in the polymorphism existing within the species (Lewontin, 1974).

Gene evolution does not only involve changes in the structure of the gene but also change its regulation. King and Wilson (1975) found human proteins to be on the average 99% identical to chimpanzee proteins and suggested that changes in gene regulation rather than changes in structural genes are the key to anatomical evolution. Two kinds of changes in DNA can alter the pattern of gene regulation, i.e. changes in nucleotide sequences coding for regulatory DNA segments and changes in the organization of genes on chromosomes. Examples of changes in regulatory DNA sequences during evolution are known in

bacteria. Most bacteria adapt to new environments by means of mutations that alter gene regulation but there is no evidence that gene regulation in higher organisms resembles that of bacteria.

The effects of increases in the number of structural genes of an organism have been investigated by analysis of the activity and electrophoretic mobility of their protein products. Carlson (1972) studied the activity of a number of enzymes in primary and secondary trisomics of Datura stramonium and demonstrated that the presence of an additional structural gene causes a marked, approximately 50%, increase in enzyme activity. He proposed that the rate of transcription is constant for each structural gene and independent of the number of identical structural genes in the genome, although the trisomic condition was often found to reduce enzyme activity. Smith and Conklin (1975) studied the effect of gene dosage on peroxidase isozymes in Datura stramonium and found no change in peroxidase activity in the tetraploid as compared to the diploid plant which suggested that the increase in structural genes was balanced by the increase in regulatory genes. An increased dosage of regulatory genes is also thought to account for the observed reduction in peroxidase isozyme activity in some trisomic plants. Zymogram analyses of compensating nullisomic-tetrasomic series of hexaploid wheat demonstrated the location of triplicate genes on homoeologous chromosomes (Hart, 1973, 1975). A linear relationship between gene dosage and quantity of the gene product suggest the same transcription rate for

structural genes of different genomes (Hart, 1975). Species in the Triticinae, however, showed increases in enzyme activity with increases in ploidy level but the increases were not proportional to the expected gene dosage effects (Mitra and Bhatia, 1971). McDaniel (1972) found a 16-30% increase in mitochondrial malate dehydrogenase activity in barley trisomic as compared to diploid plants and postulated the presence of a regulatory mechanism restricting the enzyme activity of trisomic plants. Fern gametophytes (1x, 2x and 4x) and sporophytes (2x and 4x) showed a lack of qualitative and quantitative protein differences with a change in ploidy level but displayed distinctive protein differences between the morphologically dissimilar gametophytic and sporophytic phenotypes. Peroxidase activity per cell increased in direct proportion to increases in genome and was determined to be gene dosage related. But quantitative analyses of individual peroxidase bands indicated both increases and decreases with an increase in ploidy level. De Maggio and Lambrukos (1974) concluded that the isozyme activity is controlled by regulatory mechanisms modulated by the environment.

A parallel between the rate of chromosomal changes and the rate of morphological evolution has been suggested in animals (King and Wilson, 1975). Speciation in Drosophila is known to occur without chromosomal rearrangements and chromosomal rearrangements often lead to chromosomal polymorphism rather than isolation mechanisms. Yet each Drosophila species has its own species specific chromosomal

polymorphism and only rarely do even the most closely related species share the same rearrangements (Mayr, 1970). Peripheral populations of species with polymorphism for chromosomal rearrangements show a tendency towards a reduction in the degree of polymorphism. Successful and widespread species also have less chromosomal rearrangements, presumably because the rearrangements decrease the flexibility and adaptability of the species (Mayr, 1970). Polymorphism for inversions is less common in plants than animals. Uncondensed chromosome segments (allocycly) are sometimes visualized in plants and probably reflect minute inverted chromosome segments and deficiencies. The distributional pattern of allocyclic variants of Trillium species in Japan and western U.S. was found to be correlated with differences in population size, regional and local climate and geological history (Stebbins, 1971). Peripheral populations showed less allocyclic variants.

Structural heterozygosity for translocations is highly developed in various genera of the Onagraceae. A necessary prelude to the evolution of permanent translocation heterozygotes was the presence of metacentric chromosomes with centromeres embedded in a region of heterochromatin and the occurrence of translocations at the centromere producing whole arm and equal exchanges. Hybridization of populations with different translocations, the development of lethals and self-pollination resulted in the maintenance of a system with a high survival rate in which plants are able to breed true and profit

from maximum hybrid vigor (Cleland, 1972). In most organisms, translocations have a net deleterious effect because translocated chromosomes are likely to be of unequal size as the result of unequal exchanges. Individuals heterozygous for the translocation will have irregular separation of meiotic chromosomes resulting in duplications and deficiencies and gametic inviability or sterility. Chromosomal rearrangements do change gene linkage relationships and may result in the development of adaptive gene clusters or so called supergenes or serve as effective reproductive isolation mechanisms (Stebbins, 1958, 1971).

Instantaneous speciation as the result of chromosome doubling is more widespread among plants than animals. About 30-35% of the species of flowering plants are of polyploid nature. The presence of meiotic disturbances in species hybrids and the frequent occurrence of alloploids provide evidence for the importance of structural chromosomal differentiation in plant evolution. The evolutionary advantage of polyploidy combined with hybridization lies in the stabilization of genotypes by reducing the effects of mutation, genetic segregation and recombination, elimination of the sterility of interspecific hybrids and in their higher buffering capacity against environmental changes (Stebbins, 1971).

An increase in ploidy level is found to be associated with a decrease in intraspecific variation. Bhatia et al. (1968) studied esterases, alcohol dehydrogenases and peroxidases of seed extracts of

barley and tetraploid and hexaploid wheat and found a decrease in the number of isozymic variants with an increase in ploidy level. Similar results were obtained by Waines (1969) with alcohol extractable seed proteins of Aegilops species. It suggests that only a few biotypes of each diploid hybridize to form a tetraploid species and even fewer tetraploid and diploid biotypes hybridize to form a hexaploid.

Although intraspecific isozyme variation decreases with an increase in ploidy level, polyploid species originating from chromosome doubling of species hybrids show an increased number of isozymes over their ancestral parents. Genome specific isozymes or proteins have been used successfully to study the origin of Nicotiana tabacum (Sheen, 1972), of polyploid species within the Triticinae (Johnson et al., 1967; Waines, 1969) and others. The studies demonstrated the principal of additivity of protein patterns for hybrids of known parentage and make it possible to similate hybrid protein patterns by in vitro mixture of parental proteins (Johnson et al., 1967).

The frequency of polyploidy in a plant species is affected by its growth habit and breeding system. Perennial herbs produce more polyploids than annuals and a high correlation between an efficient vegetative reproduction system and polyploidy is known to exist (Stebbins, 1971). It enables the polyploid to overcome its initial stages of irregular meiosis and semi-sterility. Polyploidy appears to be just as common in self- as cross-fertilizing perennials but is almost entirely confined to self-fertilizing annuals since self-fertilization greatly increases the chances of survival of the annual.

Drastic environmental changes followed by the opening up of new habitats have been considered as the primary stimulus for the establishment and success of polyploids. Woody genera, especially those of temperate zones, probably did not have to cope with severe environmental changes, but have in spite of their low frequency of polyploids, a relatively high basic chromosome number. Since some woody genera of tropical regions have a low basic chromosome number and very primitive floral anatomy, Stebbins (1971) concluded that the high basic chromosome number of woody genera was the result of ancient polyploidization.

Primitive plant families and orders have the greatest variation in chromosome number while morphological specialization seems to be associated with a greater stabilization in chromosome number (Stebbins, 1971). Cytological diversity seems to be related to morphological conservatism and morphological specialization with cytological homogeneity or alterations of individual chromosomes (Stebbins, 1971).

The answer to the question of how organisms evolve is complex and must await further studies of chromosomal organization and the control of gene expression in higher organisms.

Peroxidases

The enzyme peroxidase (donor: H_2O_2 oxidoreductase; E.C.1.11.1.7) uses H_2O_2 for the oxidation of a wide variety of

H-donors (AH_2) such as phenolic substances, amines, leuco-dyes, certain heterocyclic compounds like ascorbic acid and indole, cytochrome C, nitrites and inorganic ions. The reaction is as follows:

$$AH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} 2\,H_2O + A$$

Peroxidases can promote a large variety of coupled oxidation reactions and exhibit a versatility not surpassed by any other enzyme. While the enzyme is not particularly specific in its requirement for an H-donor, its specificity for peroxides is very high in that only H_2O_2, methyl- and ethyl-peroxides combine with the enzyme (Saunders, 1964).

Peroxidases are hemoproteins, which consist of an apoenzyme, that contains both carbohydrate and protein, and protohemin IX as the prosthetic group. The enzyme is thermostable, has a MW of about 40000, and exhibits one of the highest known turnover rates (Saunders, 1964).

That peroxidases exist as isozymes was first detected by Theorell (1942) in horseradish roots. It is now recognized that peroxidase isozymes are of widespread occurrence throughout the plant kingdom (Scandalios, 1974). Their number and relative concentration varies with cell and tissue type and with the developmental stage of the organ. Even in the biochemically most thoroughly studied peroxidase system, that of horseradish roots, the number of peroxidase isozymes varies from 4 to 20 (Paul and Stigbrand, 1970; Shannon et al., 1966; Delincee and Radola, 1970), depending on the extraction

technique, storage and age of the tissue. Storage in slightly alkaline conditions can alter the electrophoretic mobility of peroxidase isozymes and cause artifacts (Liu and Lamport, 1973). The major drawback of the horseradish peroxidase system is that it lacks genetic definition which complicates vigorous characterization of the isozymes developmentally and physiologically (Scandalios, 1974).

In those plant systems where genetic analysis of peroxidase isozymes has been carried out, it is apparent that the expression of peroxidase isozymes is under the control of several independently segregating genetic loci. Brewbaker and Hasegawa (1975) identified nine genetic loci with 2-6 alleles per locus in maize. Allelic variants of these loci include codominant positional alleles at 6 and null variants at 5 loci. Four variant isozymes of barley were found to be controlled by allelic genes at four loci (Felder, 1970) and Rick et al. (1974) demonstrated that the banding patterns of anodal peroxidases of red fruited tomato species were governed by codominant alleles at four loci. Alleles at three loci coded for a modified enzyme migration pattern while those at the fourth locus coded for the presence or absence of a band. Genetic studies in these and other plant species (Scandalios, 1969; Levings et al., 1971; Smith, 1972; Hoess et al., 1974; Denna and Alexander, 1975) and the biochemical investigations of horseradish peroxidases (Shih et al., 1971) indicate that peroxidases exist as monomers. Rice leaf tissue, however, showed a peroxidase locus with alleles controlling a dimer-peroxidase since

hybrid bands were found in heterozygote individuals (Shahi et al., 1969). Peroxidase alleles with multiple electrophoretic bands were observed in maize (Brewbaker and Hasegawa, 1975) and Cucurbita pepo (Denna and Alexander, 1975). These maize alleles develop three regularly spaced isozymes during tissue maturation which increase in concentration with increasing age and are thought to be under epigenetic control. The Cucurbita pepo allele controls a cluster of four bands, is codominant to an allele which produces only one band, and does not show developmental changes.

Peroxidases are known to be both temporally and spatially determined. The developmental significance of temporally specific isozymes was first appreciated by Markert and Møller (1959). Each animal tissue was found to have its own characteristic pattern of isozymes which undergoes a change during embryonic development and maturation of the organism. The principal alterations encountered are the quantitative and qualitative changes in enzyme activity (Scandalios, 1974).

Dormant seeds appear to have very little peroxidase activity, largely confined to several dead papery layers surrounding the embryo (Alexander, 1972). Upon imbibition the total peroxidase activity increases along with an increase in the number of peroxidase bands (Macko et al., 1967; Bhatia and Nilson, 1969; Alexander, 1972). Siegel and Galston (1966) examined the peroxidase pattern of rye seed germinated in H_2O or D_2O and found that one peroxidase was

synthesized before germination while five other isozymes were partially or totally synthesized during imbibition and germination. Anstine et al. (1970) demonstrated the differential expression of one genetically defined peroxidase in the in the development of barley.

Different plant organs and tissues contain characteristically different sets of peroxidase isozymes (Siegel and Galston, 1966; Felder, 1970; Alexander, 1972; Scandalios, 1974; Bonner et al., 1974). Roots are known to have relatively high and flower parts relatively low peroxidase activity (Saunders, 1964). Brewbaker and Hasegawa (1975) studied the tissue specificity of genetically defined maize peroxidases and found that, with the exception of two isozymes, all peroxidases have distinct tissue specificity with a maximum of eight loci in the coleoptile and only two in the pollen. Loy (1967) found heavy localization of peroxidases in the epidermis and xylem of Cucurbita pepo plants and observed peroxidase activity in the paravascular fibers and to some extent in the pith and cortex. Siegel (1953) found a high peroxidase activity in the xylem of kidney beans, while van Fleet (1959) and de Jong (1967) found peroxidase to be located mainly in the phloem tissue.

It is evident that plant peroxidases have a broad subcellular distribution. Not only are they found in the cytoplasm but also in association with the cell wall (de Jong, 1967; Stafford and Bravinder-Bree, 1972; Hepler et al., 1972; Raa, 1973; and others), cell

membrane (Gardiner and Cleland, 1974), nucleus (Raa, 1973), ribosomes (Darimont and Baxter, 1973; Penon et al., 1970), mitochondia (Ramaraje Urs et al., 1974), and microbodies (Plesnicar et al., 1967). Gardiner and Cleland (1974) observed that the bulk of oat and wheat coleoptile peroxidases is located in the cytoplasm and in the salt extractable cell wall fraction and that only a small fraction is tightly bound to the cell wall. Similar results were obtained by Liu and Lamport (1974) in horse radish roots; only 1.4% of the peroxidases was tightly wall-bound. Variation in the wall bound peroxidase fraction of maixe was shown to parallel cell and tissue development, with older tissues containing more wall bound peroxidases (Brewbaker and Hasegawa, 1975). Haard (1973) obtained similar results in ripening banana fruits. Two peroxidase isozymes, associated with wall particulates, increased in activity concomitantly with the respiration climateric of the fruit. The maize data suggest a general correlation between basic charge of the peroxidase isozymes and its association, largely through covalent binding, with the wall pellets. Cathodic peroxidases of sorghum were also mainly cell wall bound, while the anodic peroxidases were mainly soluble enzymes (Stafford and Bravinder-Bree, 1972). Plant and animal cell membranes are highly permeable to peroxidases which has resulted in the use of the enzyme in histo- and cyto-chemical studies (Graham et al., 1966). This also explains the presence of peroxidases in plant cell culture media (Olson et al., 1969).

One of the important reasons for the ambiguity in the interpretation of peroxidase isozyme studies is that individual peroxidase isozymes have different H-donor specificities and reactivities (Alexander, 1972; Liu, 1975; Brewbaker and Hasegawa, 1975; and others). The resolved isozymes of horseradish peroxidase exhibit specific activities which differ 10-fold when o-dianisidine is used to assay peroxidase activity. When the same isozymes were assayed for oxaloacetate oxidation, the differences in specific activity of the individual isozymes did not fit the pattern established with o-dianisidine (Kay et al., 1967). Two peroxidase isozymes of the dwarf tomato plant have different H-donor and H_2O_2 concentration requirements for maximum activity when guaiacol is used as the substrate (Evans, 1970). Heterogeneity in both electrophoretic and catalytic properties has been reported for peroxidase isozymes of many other plant species (Kawashima and Uritani, 1963; Sheen, 1974; Laurema, 1974; Brewbaker and Hasegawa, 1975), and indicates that although peroxidase isozymes may function by a similar mechanism, they mediate different reactions because of their location in different physico-chemical environments.

The variability in substrate specification of the individual isozymes underscores a basic problem of peroxidase research: lack of a clear cut physiological role for the enzyme. Different hypotheses have been put forward to explain the function of the enzyme.

Lignin Polymerase Hypothesis

Investigations on the enzymatic control of lignification (Freudenberg, 1959) have led to the suggestion that peroxidase is involved in the polymerization of p-hydroxycinnamylalcohol precursors to lignin. Support for the theory came from in vitro experiments of Siegel(1962) in which lignin like material formed on a polysaccharide matrix from a solution of eugenol, H_2O_2 and peroxidase. Peroxidase isozymes of maize (Brewbaker and Hasegawa, 1975), horseradish (Liu, 1975) and squash (Denna and Alexander, 1975) can utilize eugenol as a H-donor. Cathodal maize peroxidase isozymes were especially active on eugenol and revealed a substantial decrease in wall binding in a mutant which contained 30% less leaf and stalk lignins. Freudenberg et al. (1968), however, is sceptical about the importance of eugenol as protolignin precursor.

If peroxidase is involved in lignification, it should be localized in tracheary elements especially in secondary walls which become heavily lignified. Wall localized peroxidase has frequently been demonstrated in both cell free wall preparations and histochemical tissue preparations (Hepler et al., 1972), although de Jong (1967) found peroxidase to be associated with nonlignified tissue. Harkin and Obst (1973) used syringaldazine to demonstrate that the phenoloxidase responsible for the dehydrogenative polymerization of lignin precursors is a peroxidase.

IAA-Oxidase Hypothesis

It has been realized since the early 1950's that IAA can be oxidized by plant peroxidase (Galston et al., 1953; Goldacre, 1951), but the molecular site of IAA oxidase activity remains obscure. Sequiera and Mineo (1966) reported that fresh preparations of tobacco roots lost IAA oxidase activity but retained peroxidase activity upon storage. The thermal inactivation points and pH optima of the enzymes were different. Attempts to separate the two types of activity failed on silica gels, CM- and DEAE-cellulose and DEAE-sephadex but a major IAA-oxidase peak could be separated from peroxidase activity by SE-sephadex. Hoyle (1972) and Laurema (1974), however, were unable to confirm the presence of this peroxidase-free IAA-oxidase upon SE-sephadex elution. Pea roots were found to contain one IAA-oxidase fraction without peroxidase activity (van der Mast, 1969).

Although these results indicate that the IAA oxidase and peroxidase activity are located on different enzymes, most studies suggest that peroxidase isozymes have IAA oxidase activity (Ray, 1960; Hoyle, 1972; Gordon and Henderson, 1973; Gove and Hoyle, 1974; Brewbaker and Hasegawa, 1975). Siegel (1966) suggested that the dual catalytic functions may result from two active sites since the apoenzyme possesses IAA-oxidase activity while heme attachment is needed for the peroxidative function (Siegel, 1966; Hoyle, 1972). However, Ku et al. (1970) found that when acid butanone was used to

cleave the prosthetic group from the enzyme instead of acid acetone the apoenzyme lacked IAA-oxidase activity.

IAA oxidation is affected by phenolic compounds of high and low molecular weight, coumarins, manganese, plant acids and growth hormones (Schneider and Wightman, 1974). Plant growth hormones have never been shown to have a direct effect on peroxidase and IAA-oxidase activity in vitro, but considerable effects have been demonstrated for a wide variety of tissues. Galston et al. (1968) found that IAA did induce and repress the appearance of specific isoperoxidases in tobacco pith cell culture. Similar results were obtained in other plant species (Gordon and Henderson, 1973; Schneider and Wightman, 1974).

The intracellular location of IAA-oxidases has not yet been investigated to the same extent as that of peroxidases. IAA-oxidases of pea roots were found to be membrane bound and salt-extractable (van der Mast, 1970), and an IAA-oxidase of cabbage roots was cell wall bound (Raa, 1971).

Polyphenoloxidase Hypothesis

Dual staining abilities and similar electrophoretic mobilities of peroxidase and polyphenoloxidase isozymes suggested the location of both activities on the same enzyme (Sheen and Calvert, 1969; Srivastava and van Huystee, 1973). Van Loon (1971), however, based non-identity of the enzymes on a specific staining method for polyphenoloxidase isozymes. The polyphenoloxidase complex is best known for

its diphenoloxidase activities demonstrated upon injury and the preparation of aqueous extracts from plant tissues, but the physiological role(s) of this Cu-containing o-diphenoloxidoreductase is not clear. The enzymes appear to be either deeply imbedded or loosely bound to a variety of cellular membranes (Stafford, 1974).

Hydroxyproline Hypothesis

Cell elongation depends on the cleavage and reformation of covalent links between various wall polymers. Lamport (1970) suggested that a hydroxyproline rich glycoprotein called extensin is a controller of cell wall extensibility. Such activity is supported by the findings that a decrease in growth rate is often associated with an increase in the level of cell wall hydroxyproline (Ridge and Osborne, 1970; Cleland and Karlsnes, 1967). Hydroxyproline appears to be present in a polymer covalently linked to the cell wall via an o-glycosidic linkage to arabinose (Lamport, 1970). Yip (1964) found that horseradish peroxidase in the presence of Fe^{2+}, H_2O_2 and EDTA could form hydroxyproline from proline and that increased peroxidase concentration resulted in increased hydroxyproline formation. Liu and Lamport (1968) could isolate a hydroxyproline o-glycosidic linkage from an anionic isozyme of horseradish peroxidase. Although Shannon et al. (1966) reported the presence of this imino acid in anionic horseradish peroxidase, further work on the horseradish peroxidase system indicated that hydroxyproline represented a

contaminant fraction which could be purified away from the enzyme (Shih et al., 1971). Work by Welinder (1973) on complete amino acid sequencing of horseradish peroxidase isozymes also demonstrated the absence of hydroxyproline. Similar results were obtained by Shive and Barnett (1973) based on differential extraction and Liu and Lamport (1974) based on isopycnic equilibrium centrifugation.

Peroxidases have been implicated in ethylene synthesis, since it was found that in vitro horseradish peroxidase can catalyze the formation of ethylene from keto methylthiobutyric acid (KMTB) or methional (Yang, 1967, 1969; Ku et al., 1970), but cannot use methionine. In intact tissue however, methional is a poor ethylene precursor and KMTB is utilized 2x as fast or slower than methionine, while the peroxidase system utilized KMTB 100x more effectively than methionine. Also, mono and p-diphenols promote ethylene formation from the peroxidase system while o-diphenols are active inhibitors (Yang, 1967, 1974). In contrast, ethylene production from intact tissue was not greatly influenced by the addition of either class of phenolic substances (Gahagan et al., 1968). Finally, Kang et al. (1971) reported that there was no correlation between the amount of peroxidase in pea stem tissue and the rate of ethylene evolution.

The involvement of peroxidases in ethylene synthesis has been complicated by studies of the effects of ethylene on peroxidase isozymes. Ethylene treatment of cotton seedlings (Morgan and Fowler, 1972) and sweet potato roots (Gahagan et al., 1968; Imaseki, 1970;

Shannon et al., 1971) resulted in both qualitative and quantitative changes in peroxidase isozyme patterns.

The response of peroxidase isozymes to cut injury and abscission has also been related to their effect on ethylene synthesis.

Peroxidases are assigned a special role in the resistance of plants to diseases, this role being especially linked with peroxidase participation in the formation of a necrotic barrier on the path of infection. Peroxidase activity increases during different types of infectious diseases and is associated with both qualitative and quantitative increases in peroxidase isozymes (Rudolph and Stahmann, 1964; Andreev and Shaw, 1965; Novacky and Hampton, 1968; Chant and Bates, 1970; Curtis, 1971; and others).

The accomplishment of several of these peroxidase actions is coupled to the scavenging of H_2O_2. All respiring organisms have developed elaborate defense mechanisms against the metabolic byproducts of life supporting O_2 (Fridovich, 1975). Numerous spontaneous oxidations as well as enzymatic oxidations generate O_2^-, the superoxide radical, which can undergo a reaction with H_2O_2 and generate the reactive hydroxyl radical:

$$O_2^- + H_2O_2 \text{ -------} \Rightarrow OH^- + OH^{\cdot} + O_2$$

The reaction can be prevented or minimized by the H_2O_2 scavenging action of peroxidases and catalases. Both enzymes are widespread among respiring organisms and may have coupled this H_2O_2 scavenging action with the development of several other physiological functions.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Seed of wild Cucumis species and Cucumis melo Plant Introcudtions (PI numbers) was obtained from the U.S.D.A., A.R.S., Plant Introduction Station at Experiment, Georgia, while seed of C. hardwickii, C. sativus, and C. trigonus Plant Introductions was obtained from the U.S.D.A., A.R.S., Plant Introduction Station at Ames, Iowa. Seed of Cucumis angolensis and C. dinteri was provided by Dr. T. W. Whitaker, formerly of the U.S.D.A., A.R.S., of La Jolla, California. Table 2 shows the origin, sex expression, growth habit, and classification of the wild Cucumis species by the Plant Introduction Stations. The accessions were collected by agricultural explorers in the wild or from experiment stations and market places and are maintained as open-pollinated stocks (personal communication).

The Cucumis melo Plant Introductions originated in the following countries:

Afghanistan	China	India
Africa	Ecuador	Iran
Argentina	Egypt	Israel
Asia	El Salvador	Japan
Australia	Ethiopia	Korea
Austria	Finland	Lebanon
Belgium	France	Mexico
Burma	Greece	Morocco
Canada	Hungary	The Netherlands

Table 2. Origin, sex expression, growth habit, and classification of wild Cucumis species Plant Introductions by the U.S.D.A., A.R.S., Plant Introduction Stations.

PI Number	Species Classification	Source	Growth Habit	Sex Expression
203974	C. africanus	South Africa	perennial	monoecious
274036	"	"	"	"
282450	C. zeyheri	"	"	"
299569	C. africanus or zeyheri	"	"	"
299570	" "	"	"	"
299571	" "	"	"	"
299572	" "	"	"	"
273192	" "	"	"	"
315212	C. zeyheri	"	"	"
364473	"	"	"	"
374151	C. africanus	U.S.A.	annual	?
147065	C. anguria	Brazil	annual	monoecious
196477	"	"	"	"
233646	C. anguria or ficifolius	Ethiopia	"	"
249894	C. longipes	South Africa	"	"
249895	"	"	"	"
249896	"	"	"	"
249897	"	"	"	"
282442	C. anguria	"	"	"
320052	"	Ethiopia	"	"
364475	C. longipes	South Africa	"	"
282443	C. asper	South Africa	perennial	dioecious
193498	C. dipsaceus or leptodermis	Ethiopia	annual	monoecious
236468	C. dipsaceus	"	"	"

Table 2. Continued.

PI Number	Species Classification	Source	Growth Habit	Sex Expression
196844	C. ficifolius	Ethiopia	perennial	monoecious
203975	"	South Africa	annual	"
273648	C. ficifolius or prophetarum	Ethiopia	perennial	monoecious
215589	C. hardwickii or trigonus	India	annual	monoecious
282446	C. heptadactylus	South Africa	perennial	dioecious
275571	C. hookerii	South Africa	annual	monoecious
282447	C. leptodermis	South Africa	annual	monoecious
273650	C. membranifolius	Ethiopia	perennial	monoecious
202681	C. metuliferus	South Africa	annual	monoecious
292190	"	Transvaal	"	"
203977	C. myriocarpus	South Africa	annual	monoecious
282449	"	"	"	"
299568	C. myriocarpus or zeyheri	"	"	"
374153	C. myriocarpus	U.S.A.	"	"
179922	C. prophetarum	India	annual	monoecious
193967	"	Ethiopia	perennial	"

Table 2. Continued.

PI Number	Species Classification	Source	Growth Habit	Sex Expression
273649	C. pustulatus	Ethiopia	perennial	monoecious
280231	"	"	"	"
343699	"	Nigeria	"	"
343700	"	"	"	"
343701	"	"	"	"
282441	C. sagittatus	South Africa	perennial	monoecious
271337	C. trigonus	India	annual	monoecious
Dr. Whitaker	C. angolensis	South Africa	perennial	monoecious
Dr. Whitaker	C. dinteri	South Africa	perennial	monoecious

Nigeria	South Africa	Thailand
Pakistan	Spain	Turkey
Peru	Syria	U.S.A.
Portugal	Switzerland	U.S.S.R.
Saudi Arabia	Taiwan	West Pakistan
Senegal		

while seed from C. melo varieties was purchased from commercial seed companies.

The Cucumis sativus Plant Introductions were collected in the following countries:

Afghanistan	Germany	New Zealand
Arabia	Hungary	Philippines
Australia	India	Poland
Brazil	Iran	Spain
Canada	Iraq	Syria
China	Israel	Thailand
Czechoslovakia	Japan	Turkey
Denmark	Korea	U.S.S.R.
Egypt	Lebanon	West Pakistan
England		

while seed from cucumber varieties was purchased from commercial seed companies.

Cucumis seed was planted in flats or peat pots in a 2:1:1 mixture of soil, sand and peat and grown in a greenhouse with minimum day and night temperatures of 15 C and cooling by evapo-coolers if the temperature exceeded 21 C. Seedlings grown in peat pots were transplanted to greenhouse benches for morphological, hybridization and genetic studies. Voucher specimens and slides of the wild Cucumis species were deposited at the C.S.U. herbarium (CS) in Fort Collins, Colorado.

Pollination and Identification

The techniques of self- and cross-pollination of the wild Cucumis species are similar to those used for cucumbers and muskmelons (Whitaker and Davis, 1962). Although all wild Cucumis species are monoecious or dioecious (Table 2), muskmelons are andromonoecious, and hermaphroditic flowers require emasculation prior to cross-pollination. Removal of the anthers by forceps from a perfect flower was done 12 or more hours prior to anthesis to eliminate self-pollination. All possible self- and cross-pollinations between and within the Cucumis species were carried out to determine selfing and crossing barriers and establish the inheritance of peroxidase isozymes.

Leaf, seed, ovary, and fruit morphological characteristics were studied to identify and classify the wild Cucumis Plant Introduction accessions. Ten seeds of each PI were measured for their length, width, and thickness in mm, while the number of ovaries and fruits used per PI varied. Ovary and fruit length and width, spine length and spine frequency data were taken.

Cytological Techniques

Root tips for somatic chromosome studies were collected from seeds germinated in petri dishes or peat pots. Seeds, contact treated with a fungicide, placed on a moistened filter paper in petri dishes,

were placed for 4-7 days in a seed germinator with 30 C day and 20 C night temperatures. The root tips received a pretreatment of three hours in 0.002 M 8-hydroxyquinoline at room temperature or 21 hours in water at 0 C in order to prevent spindle formation. The root tips were fixed for 48 hours at room temperature in a 3:1 mixture of 95% ethanol and glacial acetic acid, and stained for two or more days at room temperature in 0.7% aceto-carmine. The material was squashed in dilute aceto-carmine or 45% acetic acid, and the slides were made permanent by application of a drop of a 10:1 mixture of 45% acetic acid and glycerol to the cover slip edge (Tsuchiya, 1971).

Pollen mother cells were collected from greenhouse grown plants. The one-thecous anther was used to determine the meiotic division stage of the staminate flower, and the other two two-thecous anthers were fixed, stained and squashed like somatic cells if the pollen mother cells were found to be dividing. Photomicrographs of somatic and meiotic cells were taken with the Zeiss Photomicroscope II.

Pollen morphology and fertility was determined in staminate flowers prior to or after anthesis. Pollen from three or more flowers per plant were squashed in 0.7% aceto-carmine and the number of apertures per grain and percent fertile pollen was recorded. Pollen was considered fertile if visibly filled with cytoplasm.

Enzyme Extraction

Development studies of peroxidases of a highly inbred line of Cucurbita pepo indicated that qualitative differences in peroxidase banding patterns were obtained during the first week of seedling development while the isoperoxidase banding pattern of 2-, 3- and 4-week-old seedlings were indistinguishable (Alexander, 1972).

Preliminary studies with two muskmelon varieties, Delicious #51 and Iroquois, showed similar results (Figure 1). The first peroxidase isozymes appeared three days after germination (in wooden flats under a greenhouse mist bench) and were slowly migrating anodic peroxidases. On the fourth day, two of the slower migrating bands of the Px_2 cluster (Figure 1) appeared with an additional slowly migrating band in the 6% acrylamide gel layer. The Px loci are designated by numbers with the lowest number representing the most anodic, and the highest number the least anodic peroxidases, the alleles are distinguished by capital letters and the multiple bands of the alleles by lower case letters (Rick et al., 1974; Brewbaker and Hasegawa, 1975). The slowly migrating anodic peroxidases disappeared in older seedlings, while Px_1, the fastest migrating anodic band and an additional band of the Px_2 cluster were present in 9-10-day-old seedlings. A cathodic isoperoxidase was present in 4-9-day-old seedlings but lacking in older plants. Two- to three-week-old seedlings or branches were consequently used in the survey of the

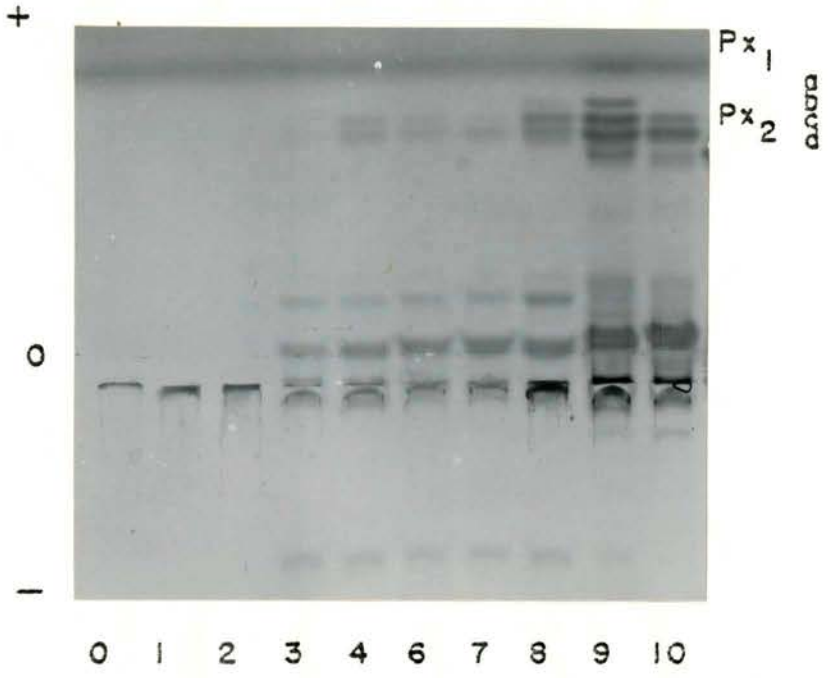

Figure 1. Peroxidases of 1-10-day-old Cucumis melo variety Iroquois seedlings.

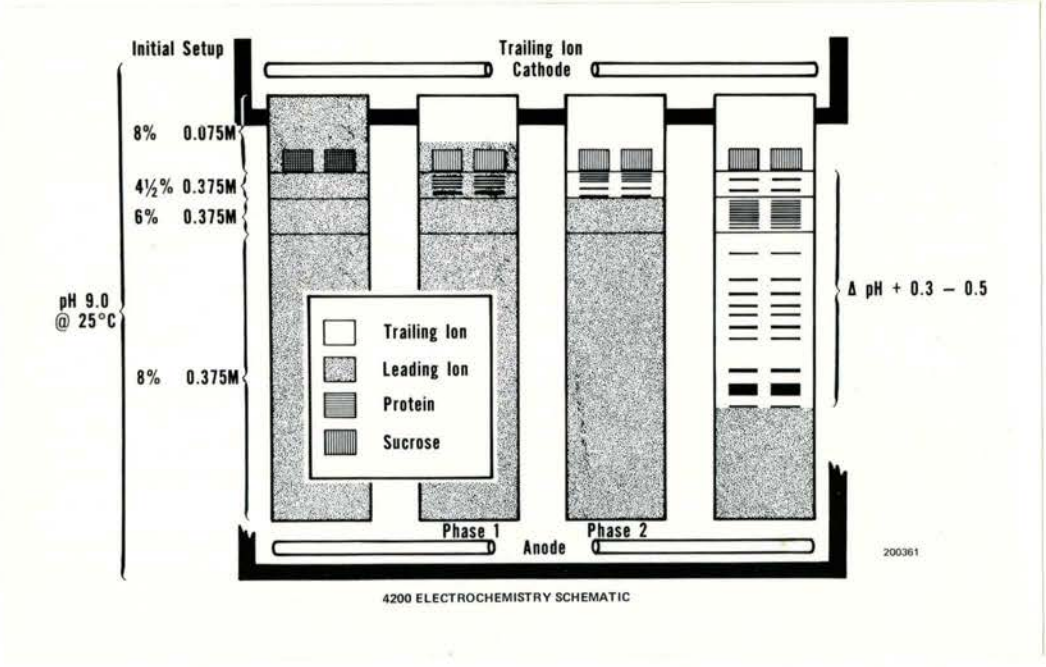

Figure 2. Performance during operation of the polyacrylamide gel electrophoresis system with discontinuous voltage gradients (Ortec Inc., Oak Ridge, TN).

Cucumis accessions and varieties. In these surveys up to 10 plants per accession or variety were harvested in bulk, because of the large number of plants and primary interest in obtaining a representative sample of peroxidase bands of the accessions.

A study of Cucurbita pepo 21- and 120-day-old plant parts showed a lack of clear cut qualitative differences in isoperoxidase banding patterns, although quantitative differences were apparent (Alexander, 1972). Studies of Cucumis melo tissues, i.e. root, hypocotyl, leaf, leaf petiole and stem, indicated the presence of Px_1 in stem tissue only, while bands of the Px_2 cluster were found in all tissues, but showed the highest activity in roots and hypocotyl. Leaf and stem tissue with or without hypocotyl was subsequently used for electrophoretic studies.

Two- to three-week-old Cucumis stem and leaf tissue was macerated with a mortar and pestle, squeezed by hand and centrifuged for 10 minutes at 1,800 x g or for 2-4 minutes at 8,000 x g. Approximately 20 µl of the supernatant, mixed in a 1:1 ratio with 80% or 50% sucrose, depending on the sample concentration, was used as sample for electrophoretic studies.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was used for the separation of proteins of the Cucumis species. The Ortec Model 4200 slab acrylamide electrophoresis system with the Model 4100 pulsed

constant power supply was used (Figure 2, page 50). The system combines the method of sample concentration by a conductivity shift resulting from ionic strength differences between the sample and separating gel (Hjerten et al., 1965) with the moving boundary method of disc electrophoresis using discontinuous voltage gradients to produce zone sharpening, and does not require pH discontinuities. The pulsed power supply system results in shorter separation times and higher resolution without denaturation (Ortec, 1972, Models 4010/4011 Electrophoresis System, Operating and Service Manual). Detailed methodology for casting polyacrylamide gel slabs for use with the Ortec apparatus are described in Ortec's 1972 publication AN 32A. The Ortec procedure was initially modified to enable the concurrent separation of anodic and cathodic proteins (Alexander, 1972). The 8% (4 cm) acrylamide layer in pH 9.0, 0.375 M Tris-sulfate buffer was immediately overlayered with a 6% (1 cm) acrylamide gel solution of pH 9.0, 0.375 M Tris-sulfate buffer. A $4\frac{1}{2}$% layer (0.5 cm in height, in pH 9.0, 0.375 M Tris-sulfate buffer), added after the polymerization of the 8-6% separating gel, constituted the stacking gel. The sample wells were formed in an 8% (1 cm) acrylamide, pH 9.0, 0.075 M Tris-sulfate gel, with a 12-well template resulting in 4 mm wide wells. The cathodic gel consisted of an 8% acrylamide solution in a pH 9.0, 0.375 M Tris-sulfate buffer. Acrylamide and methylene-bis-acrylamide were purchased from Polysciences. Since cathodic peroxidase isozymes were only occasionally observed in Cucumis

plant material, Ortec's procedure for the separation of anodic proteins was followed since it required less time and resulted in less protein diffusion. This method uses a 5.9 cm 8%, 0.9 cm 6% separating gel, 0.5 cm stacking gel, and 1 cm 8% well-forming gel. The constant pulsed power supply system with one or two cells immersed in cold, pH 9.0, 0.065 M Tris-borate buffer was operated according to Ortec's specifications, but the maximum voltage for double cell operation was reduced from 400 to 350 V.

Peroxidases were visualized by imbedding the gel slabs in a 3:1 mixture of 10^{-3} M o-tolidine and 10^{-2} M H_2O_2, since o-tolidine, compared with other H-donors, was found to be the most sensitive peroxidase substrate (Loy, 1967; Alexander, 1972).

The Cucumis melo and Cucumis sativus PI's and varieties were analyzed only once for their isoperoxidase banding pattern, but the wild Cucumis species were analyzed at least three times and their isoperoxidase bands are recorded as frequencies.

Genetic data of peroxidase isozyme pattern differences were subjected to X^2-analyses. Genotype and gene frequencies were calculated for the observed isoperoxidase banding patterns in C. melo and compared with the fruit shape index genotype and gene frequencies, calculated from data published by the Plant Introduction Station (Corley, 1966).

The staining mixture for esterase isozymes consisted of 75 mg fast blue RR salt and 40 mg α-napthylacetate, in 2 ml acetone/2 ml

water, per 100 ml 0.2 M, pH 7.0 phosphate buffer, modified after Markert and Hunter (1959). In order to visualize the esterase isozymes, the Cucumis samples had to be reduced in volume.

Glutamate oxaloacetate transaminases (GOT) isozymes were visualized according to Shaw and Prasad (1970), malate and glutamate dehydrogenases according to Honold et al. (1966), while α-amylases were made visible after the incorporation of 2% starch in the Tris-sulfate buffers and staining in the iodine reagent (0.1 N potassium iodide in 0.01 N iodine).

Since α-esterase banding patterns were not very reproducible, and the dehydrogenases and α-amylases failed to show clear-cut interspecific differences, only the GOT isozyme patterns were used.

Isoelectric Focusing

Isoelectric focusing separates proteins according to their isoelectric point rather than size and charge as in polyacrylamide gel electrophoresis or PAGE. The technique, performed in Ortec's electrophoresis system and described in Ortec's publication AN 32A, was followed with slight modifications in that a solution of 1.4 ml TEMED in 25 ml water was used and that the water volume was reduced accordingly to obtain the described TEMED concentration. Approximately 10-15 μl Cucumis sample in a 1:1 mixture with 50% sucrose was used per sample well. A solution containing 2 ml concentrated H_2SO_4 per liter H_2O was used at the anodal buffer

reservoir, and 8 ml monoethanolamine per liter H_2O at the cathodal reservoir if the ampholines had a pH range of 3.5-10.0, or a 0.065 M, pH 9.0, Tris-borate buffer if the ampholines had a pH range of 3.5-5.0. The ampholines were purchased from LKB. Ortec's pulsed power settings were followed. Peroxidases were visualized by incubating the gel slabs in a solution of 10^{-3} M o-dianisidine and 10^{-2} M H_2O_2 (1:1, v/v) since the oxidation products of o-tolidine were found to be too pH dependent.

Isolation and Purification of Cucumis Peroxidases

In order to determine efficient purification procedures, preliminary experiments were necessary to develop an accurate and sensitive quantitative peroxidase assay method, determine the effects of polymers and reducing agents on the phenol content, and the effects of temperature, pH, and salt on the isolation of Cucumis peroxidases.

Peroxidase Assay Method

Different isoperoxidases show different H-donor specificities (Jermyn and Thomas, 1954; McCune, 1964; Evans, 1968; Liu, 1975) and different reactivities towards the same substrate. Loy (1967) tested the substrate specificity of muskmelon stem peroxidases toward eight H-donors and observed the highest number of anodic and cathodic electrophoretic bands if o-tolidine or p-amino-3-methoxydiphenylamine were used. O-tolidine was subsequently used

for quantitative peroxidase determinations in that the change in absorbance at 635 nm due to the o-tolidine oxidation by the enzyme in the presence of H_2O_2 was measured spectrophotometrically. Salomon and Johnson (1959) found a relatively narrow absorbance band of oxidized o-tolidine at 365 nm and a broad band at 635 nm with the sensitivity of the broad band being 68% lower than that of the narrow band. The change in absorbance per minute per μg purified horseradish peroxidase (NBC) was followed spectrophotometrically (B&L, Spectronic 20) at 365 and 635 nm.

△ 0D/min/μg HRP	wavelength in nm
0.83 ± 0.09*	365
0.57 ± 0.03	635

*Indicates the standard error of the estimate.

The reaction medium consisted of 4.8 ml, 0.1 M acetate buffer, pH 5.0, containing 10^{-4} M o-tolidine, 0.1 ml of 5×10^{-3} M H_2O_2, and 0.1 ml horseradish peroxidase in concentrations ranging from $1\text{-}10^{-2}$ μg. The results confirmed Salomon and Johnson's observations. Peroxidase activity was consequently measured at 365 nm if o-tolidine was used as the H-donor.

Since Loy (1967) did not include o-dianisidine in the H-donor specificity test of muskmelon peroxidases, and Alexander (1972) rated o-dianisidine as a good substrate for Cucurbita pepo peroxidases, its specificity towards purified HRP was compared with that of o-tolidine. The reaction mixture consisted of 0.1 ml HRP in concentrations

ranging from $1\text{-}10^{-1}$ µg, 2.75 ml pH 5.5, 0.05 M acetate buffer, 0.05 ml 20 mM o-tolidine or 20 mM o-dianisidine and 0.1 ml 4 mM H_2O_2. The change in absorbance at 365 nm for o-tolidine and 460 nm for o-dianisidine (Shannon et al., 1966) was followed spectrophotometrically.

Δ 0D/min/µg HRP	substrate
0.44 ± 0.01*	o-dianisidine
0.87 ± 0.05	o-tolidine

*Indicates the standard error of the estimate.

The results indicate that o-tolidine provides a more sensitive peroxidase assay than o-dianisidine.

Peroxidase activity measurements are known to be pH dependent (Saunders, 1964) and since different plant species have different peroxidases, the pH optimum for cucumber peroxidase activity measurements was determined. Using a 0.05 M acetate buffer of pH 4.0, 4.5, 5.0 or 5.5, the change in absorbance at 365 nm due to the peroxidase oxidation of o-tolidine in the presence of H_2O_2 was followed spectrophotometrically. The reaction medium consisted of 2.75 ml, 0.05 M acetate buffer, 0.05 ml 20 mM o-tolidine, 0.1 ml 4 mM H_2O_2, and 0.01ml cucumber juice. The juice was obtained by grinding 1 g fresh cucumber material in 1 ml H_2O, centrifuging it for two minutes at 8, 000 x g and diluting the supernatant with water.

Δ OD/$\frac{1}{2}$ min/mg cucumber plant material	pH
0.11	4.0
0.15	4.5
0.29	5.0
0.20	5.5

The results indicate the optimum pH of 5.0 for cucumber peroxidase activity determination. The following reaction mixture was subsequently used for peroxidase activity determinations:

2.75 ml, 0.05 M acetate buffer, pH 5.0

0.05 ml 20 mM o-tolidine

0.1 ml 4 mM H_2O_2

0.1 ml or less sample

and the change in absorbance measured at room temperature after $\frac{1}{2}$ minute at 365 nm against a solution without o-tolidine as standard.

Protein Determination

Lowry's method (Lowry et al., 1951) was used for protein determinations. Alpha Globulin, fraction IV (Pentex Inc.) was used as a standard.

Reduction of Phenol Oxidation

Since phenols are known to reduce the Folin-Ciocalteau reagent, experiments were done to remove phenolics from Cucumis juice and/or prevent their oxidation. The oxidation of phenols to quinones followed by covalent coupling reactions or oxidation of reactive protein groups are the principal basis of browning reactions in plant tissues and extracts. The oxidation is catalyzed by phenol oxidases and

occurs if tissue is homogenized because phenolics are spatially separated from phenol oxidases in intact tissue. Phenolics can also form H-bonds with the O-atoms of peptide bonds, salt linkages with basic amino acid residues, especially at pH values higher than 8.5 and hydrophobic interactions (Anderson, 1968; Loomis, 1973).

Polymers, like polyvinylpyrrolidine (PVP), are known to inhibit purified o-diphenoloxidase preparations and bind those phenolics that form strong H-bonded complexes (Loomis, 1973).

The adsorption spectrum of compounds found in brown colored muskmelon juice was determined spectrophotometrically (Figure 3). Frozen muskmelon plants were macerated with a mortar and pestle, centrifuged for 2-4 minutes at 8,000 x g and left for 12 hours at room temperature. The brown colored juice shows a maximum absorbance at 365 nm.

The effect of insoluble PVP, Polyclar AT (GAF Corporation), purified according to Loomis (1973) to remove metal ions and the vinylpyrrolidine monomer, on preventing the brown coloration of muskmelon juice and peroxidase activity was determined. One gram frozen muskmelon tissue was macerated in 1 ml water with a mortar and pestle, immediately centrifuged at 8,000 x g for two minutes, after which insoluble hydrated PVP was added to the supernatant which was allowed to oxidize for 12 hours at room temperature. The absorbance per ml muskmelon juice (supernatant) was determined at 365 nm, and the peroxidase activity was measured as described earlier.

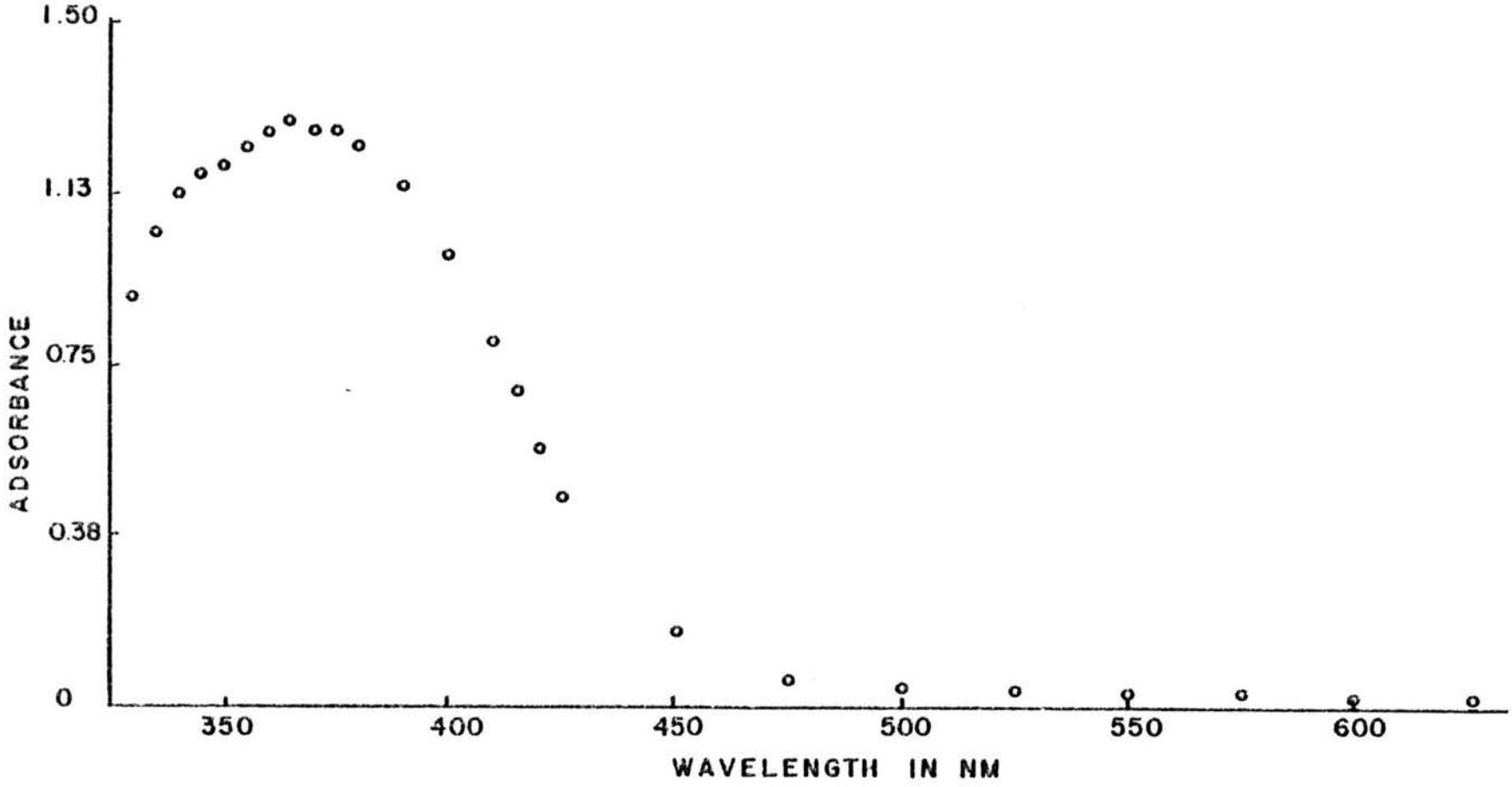

Figure 3. Adsorption spectrum of compounds responsible for the brown coloration of muskmelon plant extract.

g PVP per ml juice	OD at 365 nm/.01 ml juice after 12 hours	peroxidase activity Δ OD/$\frac{1}{2}$ min/.01 ml juice
0	.52 ± .01*	.26
0.001	.47 ± .01	.27
0.01	.43 ± .01	.30
0.1	.19 ± .01	.27

*Standard deviation values.

The results indicate that PVP reduces the formation of brown coloration of muskmelon juice, but has no effect on peroxidase activity.

The formation of oxidation products can also be prevented by inhibition of phenol oxidases. Metabisulfite and 4-chlororesorcinol are known as phenol oxidase inhibitors (Anderson and Rowan, 1967; Stokes et al., 1968; Loomis, 1973). Kull et al. (1954) tested a large number of substrate analogs for possible inhibition of potato polyphenol oxidase and found 4-chlororesorcinol to be very effective with inhibition already detectable at a concentration of 10^{-8} M. Boric acid is known for its ability to form soluble complexes with polyols, including o-diphenols (Weser, 1968; King, 1971) and is effective at 0.2 M concentration (King, 1971).

The effect of these compounds on brown coloration of muskmelon juice and peroxidase activity was determined. Muskmelon juice was obtained by macerating 5 g frozen tissue in 5 ml 0.1 M boric acid, 5 ml 0.01 M metabisulfite, or 5 ml 10, 100, or 500 mM 4-chlororesorcinol.

Phenol oxidase inhibitor	concentration per g tissue	OD at 365 nm/ 0.01 ml juice after 12 hours	peroxidase activity Δ OD/$\frac{1}{2}$ min/0.01 ml juice
boric acid	1 ml 0.2 M	0.09	0.30
metabisulfite	1 ml 0.01 M	0.08	0.10
control	1 ml H_2O	0.08	0.39
		After 16 hours	
4-chlororesorcinol	1 ml 0.01 M	0.046$\pm$0.005*	0.10
	1 ml 0.1 M	0.048$\pm$0.002	0.07
	1 ml 0.5 M	0.051$\pm$0.001	0.09
control	1 ml H_2O	0.045$\pm$0.003	0.14

*Standard deviation values.

The results indicate that metabisulfite, boric acid and 4-chlororesorcinol do not reduce the brown coloration of muskmelon juice, but do reduce the peroxidase activity considerably. The difference in results of the boric acid and metabisulfite experiments and those of 4-chlororesorcinol are due to differences in muskmelon plant sample and a difference in oxidation (incubation) time (16 versus 12 hours).

Effect of Temperature on Cucumis Peroxidase Activity

Temperature near and above boiling are required to destroy peroxidase activity in most plant tissues and extracts (Wilder, 1962). Peroxidase isozymes show differences in heat stability (Kon and Whitaker, 1965) and Gordon and Alldridge (1971) found the heat inactivation temperature to be dependent on the pH of the extraction medium.

Extraction of peroxidases from cucumber tissues in an acetate buffer (pH 5.0), (w/v=1:1) at 4 C and room temperature resulted in less enzyme activity at the lower extraction temperature.

Extraction Temperature	Peroxidase activity in Δ 0D/$\frac{1}{2}$ min/.01 ml juice	
	immediately after extraction	18 hours after extraction
4 C	0.27 $\pm$ 0.05*	0.36 $\pm$ 0.05
20 C	0.42 $\pm$ 0.08	0.38 $\pm$ 0.07

*Standard deviation values.

Heat treatment of cucumber juice on a waterbath was found to have many advantageous effects in that not only most of the other proteins precipitated, but also the peroxidase activity was elevated.

Heat Treatment	Peroxidase activity in Δ 0D/$\frac{1}{2}$ min/.01 ml juice
control	0.64 $\pm$ 0.01*
5 min at 70 C	0.78 $\pm$ 0.11
10 min at 75 C	0.82 $\pm$ 0.17

*Standard deviation values.

Electrophoretic analysis of extracts from other heat treated Cucumis plant material revealed the absence of peroxidase band Px_1 upon treatment for 2 or 5 minutes at 70 C, while a treatment of 5 minutes at lower temperatures (arbitrarily chosen 50 C) had no effect on Px_1. Consequently, a treatment of 5 minutes at 50 C was used for tissues of all Cucumis species except C. sativus which was treated for 5 minutes at 70 C.

Effect of pH and Salt on Cucumis Peroxidase Extraction

Peroxidases are known to differ in extractibility depending on the subcellular distribution of the enzyme (Lee, 1971; Denna and Alexander, 1975). Lee (1971) found a medium of high ionic strength essential for extraction of peroxidase isozymes of tobacco callus tissue. The salt extractable enzymes were slowly migrating anodic peroxidases (Lee, 1971; Gordon and Alldridge, 1971). Alexander (1972) found two weakly bound Cucurbita pepo peroxidases to be released by salt (1 M NaCl), 8 M urea or 0.3% cholate, while cellulase released a small amount of peroxidase activity, which produced mainly diffuse zones of activity in the gels near the anodic and cathodic front.

Experiments were done to determine the effect of pH of the extraction medium, salt and cellulase digestion on cucumber peroxidase activity and isozyme pattern. Cucumber tissue (2 gram) was macerated in 2 ml 0.05 M acetate buffer of pH 5.0 or 2 ml 0.05 M P-buffer, pH 6.0, and centrifuged at 15,000 x g for 10 minutes. The pellet was resuspended in 5 ml buffer of pH 5.0 or 6.0, recentrifuged and resuspended until the supernatant lacked peroxidase activity. Weakly bound peroxidases were removed by treating the pellets with 5 ml buffer of pH 5.0 or 6.0 containing 0.8 M KCl, centrifugation at 20,000 x g for 10 minutes and resuspending in 5 ml 0.8 M KCl, pH 5.0 or 6.0 buffer. When the supernatant lacked peroxidase activity, the pellet was incubated for 20 hours in the pH 5.0 or 6.0 buffer

containing a few grains of cellulase (Worthington Biochemicals). The peroxidase banding pattern of salt and cellulase treated solutions was found to contain no new isoperoxidases. Salt treatment, however, greatly increased the amount of peroxidase released, as did incubation in a low pH extraction medium.

Treatment	buffer pH	peroxidase activity Δ 0D/$\frac{1}{2}$ min/.01 ml juice
cucumber supernatant		
	5.0	0.66 $\pm$ 0.07*
	6.0	0.30 $\pm$ 0.06
buffer washes (3x) of the pellet		
	5.0	.80
	6.0	.44
salt washes (2x) with 0.8 M KCl in buffer		
	5.0	.54
	6.0	.42
cellulase digestion in buffer		
	5.0	.10
	6.0	.03

*standard deviation

It should be noted, however, that the difference in peroxidase activity measurements at pH 5.0 versus 6.0 may have been the result of differences in buffer and the low pH optimum of cellulase.

Purification of Cucumis Peroxidases

Cucumis plant material (without roots) was macerated with a meat grinder and based on preliminary experiments, hydrated insolubel PVP (0.1 g per ml juice) and KCl (0.8 M) were added immediately. The ground material was squeezed through three to four layers of cheesecloth, the retained fibrous material resuspended in a

minimal volume of 0.05 M, pH 5.0 acetate buffer, containing 0.8 M KCl, squeezed through cheesecloth and discarded. The juice was subsequently heated on a waterbath for five minutes at 50 C or 70 C (C. sativus), cooled, and centrifuged for 10 minutes at 20,000 x g.

The peroxidases were further purified by ammonium sulfate fractionation (Shannon et al., 1966), using a 30% initial saturation level (1.8 g/ml). After standing overnight at 4 C, the solution was centrifuged at 20,000 x g for 15 minutes and the supernatant was brought to 85% saturation with $(NH_4)_2SO_4$ (4 g/ml). The residue was collected after 16 hours at 4 C by centrifugation (at 20,000 x g for 15 min), dissolved in a minimal volume of 0.05 M, pH 5.0 acetate buffer and dialyzed against this buffer until salt free (checked with $HgCl_2$).

This so-called $(NH_4)_2SO_4$ fraction was dialysed against 0.005 M Tris-HCl buffer, pH 8.0, centrifuged for 20 minutes at 20,000 x g, and loaded on a DEAE-Bio-Gel A column (Biorad Laboratories), equilibrated with the same buffer. The peroxidases were eluted from the column by a stepwise increase (0.025 M) in NaCl to a final concentration of 0.1 M NaCl in 0.005 M Tris-HCl, pH 8.0, buffer.

The volume of the peroxidase solutions was reduced by a variety of methods depending on the amount, i.e. ultrafiltration (Amicon, Diaflo ultrafiltration membrane PM-10), vacuum or sucrose dialysis and lyophilization.

Immunological Techniques

The peroxidase solution of Cucumis sativus plants purified as described above (containing all three Px_2 bands) was dialyzed against 0.15 M NaCl, mixed by syringe with Freunds complete adjuvant (v/v= 1:1) (Miles Laboratories), and injected intramuscularly in a New Zealand white rabbit.

The injection and bleeding schedule was as follows:

Day 0	Day 1	Day 15	Day 29	Day 67
bleeding normal rabbit serum NRS	IM injection of 3.5 mg protein in Freunds complete adjuvant	bleeding serum I IM injection of 7 mg protein in Freunds complete adjuvant	bleeding serum II	bleeding serum III

Bleeding was done from the marginal right ear vein and after clot formation overnight at 4 C, serum was collected and freeze dried or frozen until used.

The classic ring precipitin test, performed in 1 mm tubes (Crowle, 1973), was used to determine the optimal precipitating proportion of antigen, the $(NH_4)_2SO_4$-peroxidase fraction of the Cucumis species, with serum to the C. sativus peroxidases. The optimal antigen concentration, determined as described above, was mixed with serum (v/v=1:1) and allowed to react overnight at room temperature. The solution was centrifuged for four minutes at 8,000 x g and the supernatant was mixed with 50% sucrose (v/v=2:1) and

electrophoresed. The control solutions, which contained water instead of serum (v/v=1:1) were treated similarly. The serum to the C. sativus peroxidases precipitated peroxidases of the Px_1, Px_2 and Px_3 locus of:

C. aculeatus	C. metuliferus
C. africanus (PI 275571)	C. myriocarpus (PI 299568)
C. dipsaceus	C. sagittatus
C. figarei	C. sativus
C. leptodermis	C. anguria (PI 233646,282442)
C. melo	C. zeyheri (PI 299569,273192)

as was indicated for some species in Figure 4.

The micro immuno diffusion technique (Crowle, 1973) was used to determine the identity or partial identity relationships among the Cucumis peroxidases. The diffusion medium consisted of 1.5% purified agar (Difco Laboratories) in deionized water. The reactants were allowed to diffuse for two days at 4 C, rinsed for two days in water, stained with 0.1% thiazine red (Allied Chemicals) in 2% acetic acid, and destained in 2% acetic acid. The slides were enlarged and contact printed.

Biochemical Studies of Individual Cucumber Peroxidases

The $(NH_4)_2SO_4$-fraction of Cucumis sativus peroxidases was subjected to preparative PAGE to separate the individual isozymes. A $7\frac{1}{2}$% separation gel of 6.8 cm in pH 9.0, 0.375 M Tris-sulfate buffer, $4\frac{1}{4}$% stacking gel of 0.5 cm in the same buffer, and 8% sample gel of 1 cm in pH 9.0, 0.075 M Tris-sulfate buffer with a preparative

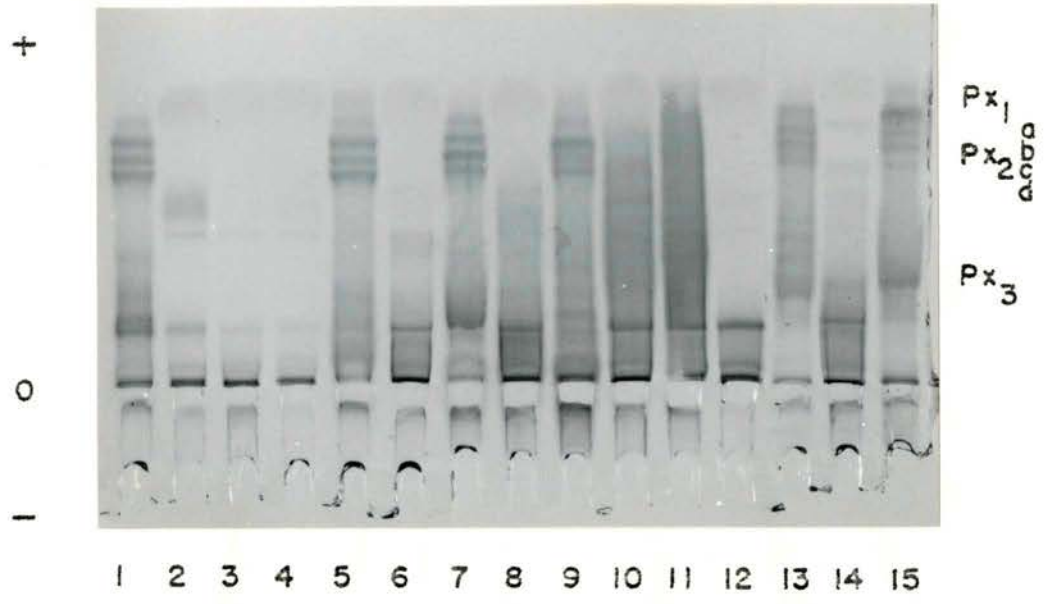

Figure 4. Peroxidases of C. sativus, C. melo, C. metuliferus, C. dinteri, C. anguria and C. aculeatus treated with or without serum to the C. sativus peroxidases.

1. C. sativus control
2. C. sativus + serum I
3. C. sativus + serum II
4. C. sativus + serum III
5. C. sativus + NRS
6. C. melo + serum III
7. C. melo control
8. C. metuliferus + serum III
9. C. metuliferus control
10. C. sagittatus + serum III
11. C. sagittatus control
12. C. anguria + serum III
13. C. anguria control
14. C. aculeatus + serum III
15. C. aculeatus control

well former were used (Ortec, 1972). The peroxidases, migrating as reddish-brown bands, were cut from the gel, after which the gel slices were homogenized and the individual peroxidases were extracted in deionized water.

The three peroxidases (Px_2 b, c and d) were subjected to several treatments: lyophilization with resuspension in water; heat treatment of five minutes at 70 C; 8 M urea; 2 M NaCl; 3% cholate; or 3% SDS (sodium dodecyl sulfate) after which the treated isozymes were electrophoresed. If the isoperoxidases are conformational isozymes, differing only in tertiary structure, reversible denaturation should generate the whole isozyme pattern for each of the original isozymes (Epstein and Schechter, 1959). However, although low concentrations of additional bands (e.g. b and c in case of peroxidase d) were observed after staining, the individual peroxidases were present in the highest concentration and visible without staining. The results indicate a lack of interconversion of the isozymes upon treatment with agents affecting secondary protein bonds and denaturation, but the treatments may not have resulted in complete denaturation.

Polyacrylamide disc electrophoresis, modified after Davis (1962), was used to determine if the three cucumber isoperoxidases were differing in size and/or charge. Separating gels of 4, 6, 8, 10 and 12% acrylamide in a Tris-HCl buffer and 2.5% spacer gel in a Tris-phosphate buffer were used. Cylindrical glass tubes (6.5 x 0.5 cm) were used with the Canalco disc electrophoresis system. The

system was operated for two hours at 1 mA/tube and one hour or longer at 2 mA/tube. The relative electrophoretic mobility or Rm value (distance traveled by the enzyme over distance traveled by the bromophenol blue front) was calculated for the three isoperoxidases. Plotting of log (Rm x 100%) versus the gel concentration resulted in parallel lines (the slopes of which were determined by linear regression analysis), indicating that the peroxidases differ in charge (Maurer, 1971) (Figure 5). The cause of the charge differences remains to be investigated.

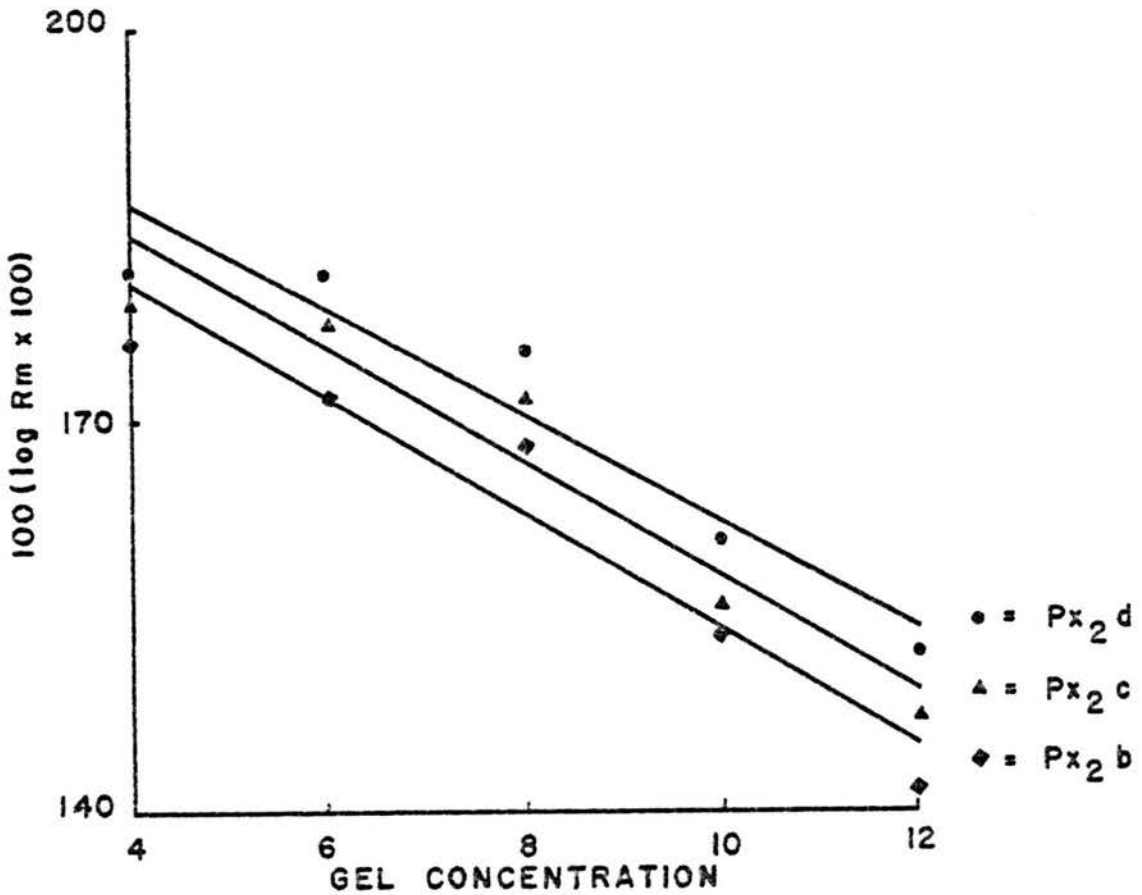

Figure 5. The relationship between the relative electrophoretic mobility of the C. sativus peroxidase isozymes and gel concentration.

RESULTS AND DISCUSSION

Cultivated Cucumis Species

Muskmelon or Cantaloupe (Cucumis melo)

Cucumis melo Plant Introductions and varieties display a characteristic peroxidase banding pattern with a fast migrating anodic peroxidase (Px_1) and a cluster of three or four slower migrating anodic bands in their leaf and stem tissues. Only three qualitatively different peroxidase patterns were observed in this species, caused by genetic polymorphism of alleles at the Px_2 locus. The Px_{2A} allele is represented by peroxidase bands a, b and c, and the Px_{2B} allele by bands b, c and d. Heterozygotes show all four (a, b, c and d) bands (Figure 6). Monogenic inheritance could be demonstrated for the alleles at the Px_2 locus. The observed genotypes showed the expected 1:2:1 ratio, and the backcross generations the expected 1:1 ratio (Table 3). Codominant inheritance of variant peroxidase alleles has been demonstrated in a variety of other plant species at a relatively large number of peroxidase loci (Felder, 1970; Rick et al., 1974; Brewbaker and Hasegawa, 1975).

In order to understand the evloutionary phenomena in natural populations the pattern of genetic variation in populations from different ecogeographic regions of the species range has to be determined

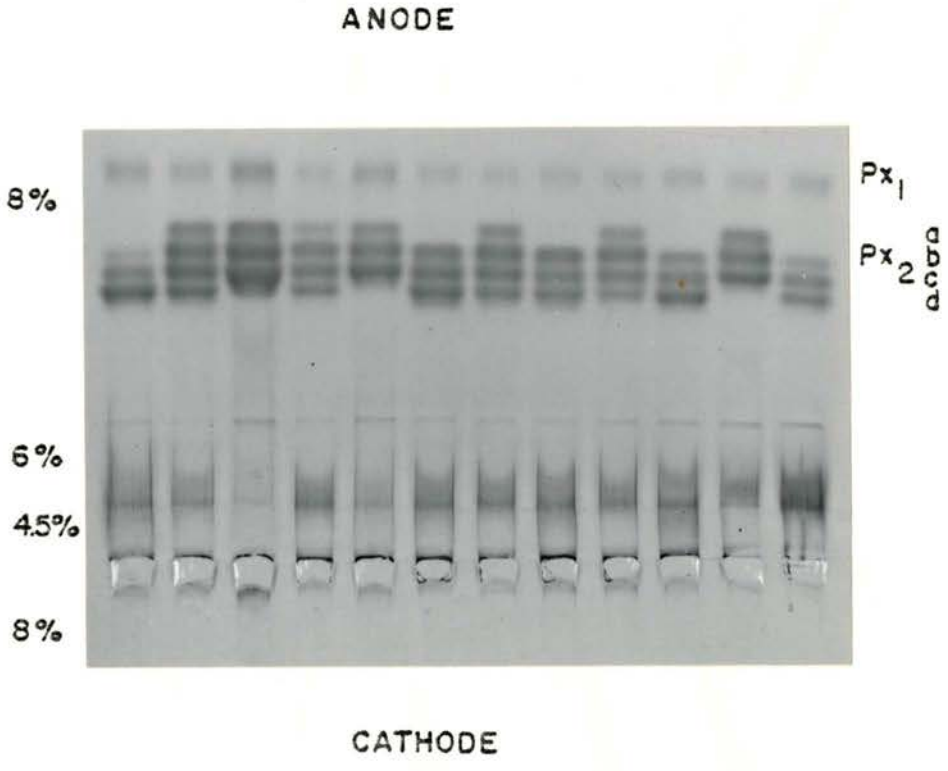

Figure 6. Peroxidase banding pattern of an F_2 generation of Cucumis melo plants.

Table 3. Segregation of F_2 and BC generations for peroxidase alleles at the Px_2 locus in Cucumis melo.

Generation	Genotypes			Total	X^2	df
	$Px_{2A}Px_{2A}$	$Px_{2A}Px_{2B}$	$Px_{2B}Px_{2B}$			
$F_1 \times Px_{2A}Px_{2A}$	11	10		21	.05	1
$Px_{2A}Px_{2A} \times F_1$	6	6		12	.00	1
Subtotal	17	16		33	.05	2
F_2	13	36	12	61	2.02	2
F_2	10	30	17	57	1.88	2
Subtotal	23	66	29	118	3.90	4

(Lewontin, 1974). The distribution of the Px_2 alleles in C. melo Plant Introductions from various countries was studied and if the peroxidase pattern of 10 or more PI's was known, their gene or allele frequency:

$$P_{Px_{2A}} = \frac{2\,Px_{2A}Px_{2A} + Px_{2A}Px_{2B}}{2N}$$

was calculated. Although the overall gene frequency, calculated from the genotype data from all C. melo PI's and varieties is 0.51, differences in gene frequencies do exist between populations from different countries (Appendix Table 1; Figures 7,8). Many accessions from India and Iran have the $Px_{2A}Px_{2A}$ genotype and a high (p=0.61) gene frequency, while 50% or more of the PI's from Turkey, Syria, the U.S.S.R., and China are $Px_{2B}Px_{2B}$ and have a low (p=0.29) frequency for the Px_{2A} allele.

Other characteristics of these accessions like fruit shape, size, surface, and color, flesh color, taste and depth, and disease resistance were analysed to determine if the geographic variation in peroxidase isozyme pattern was associated with a geographic variation in other plant characteristics.

Fruit shape measurements (Appendix Table 2), expressed as length in cm over width in cm or fruit shape index, indicated increased levels of genetic variability in Iran, India, and Turkey over Afghanistan and China. Genotype and gene frequencies of round (L/W, or fruit shape index, =1), oblong (1<L/W<2) and elongate

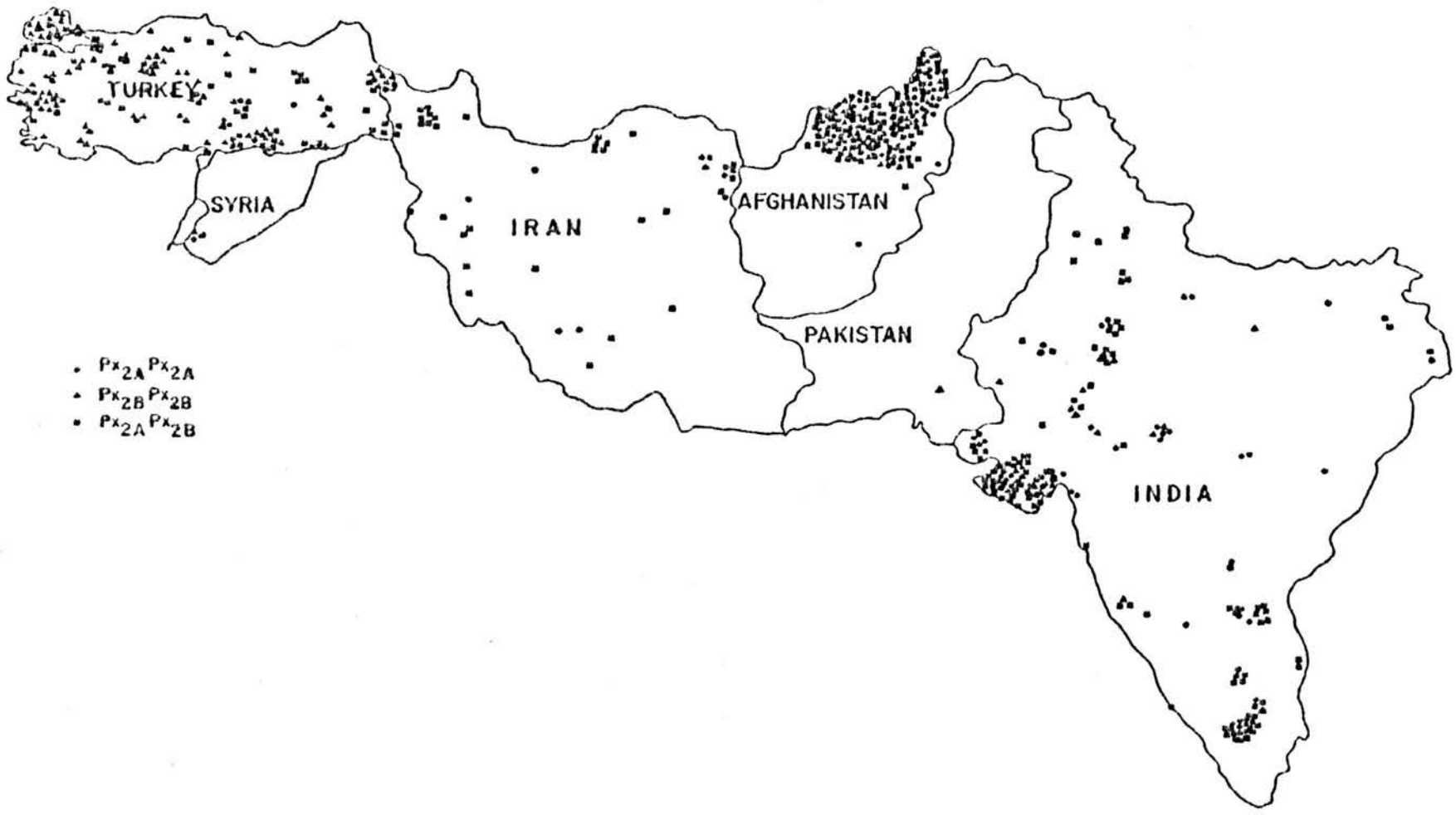

Figure 7. Geographical distribution of C. melo peroxidase alleles at the Px_2 locus.

Figure 8. Geographic variation in Px_2 allele frequency of Cucumis melo Plant Introductions.

(L/W$\geq$ 2) fruits were calculated based on Bains and Kang's (1963) genetic studies. The overall gene frequency:

$$P = \frac{2(\text{round fruited melons}) + (\text{oblong fruited melons})}{2N}$$

is 0.51, and little to no differences were found in gene frequency among PI's from Afghanistan (p=0.51), India (p=0.48), Iran (p=0.53), Turkey (p=0.52) and U.S.S.R. (p=0.59) (Figure 9). The low coefficient of variability for fruit shape index in Afghanistan and China could be attributed to a high level of heterozygosity (75%) for alleles controlling fruit shape (Appendix Table 3).

Most fruits had a smooth (37% of the PI's), furrowed (31%), or netted (18%) surface, although more than 40% of the melon fruits from Turkey had a wrinkled fruit surface (Appendix Table 4). Sixty percent of all fruits had a yellow fruit color, but, while 26-34% of the accessions from Afghanistan, India, and Iran have orange fruits (Appendix Table 5), 26% of Turkey's PI's had a green fruit color. These differences may be an indication of an increased level of homozygosity in collections from Turkey, but conclusions cannot be made because of a lack of adequate genetic studies of fruit color and surface.

Disease and insect resistance was found mainly in collections from Egypt, Syria, Turkey, India and China. India appears to be the best source of C. melo plants with resistance to powdery and downy mildew, gummy stem blight, anthracnose and Macrosporium (Figure 10; Appendix Table 6). Whitaker and Bohn (1954) found genes with

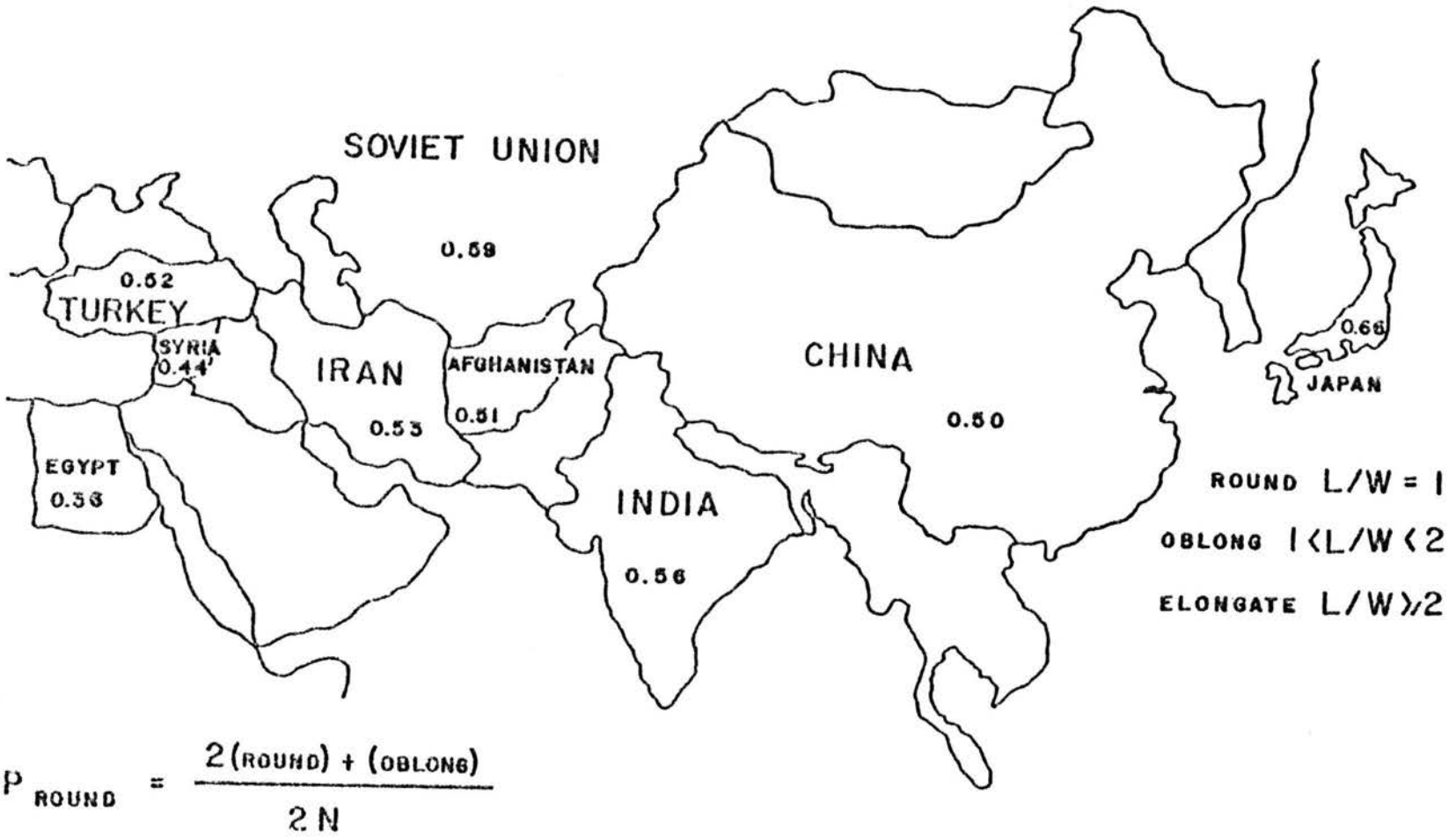

Figure 9. Geographic variation in the frequency of alleles controlling the shape of Cucumis melo fruits.

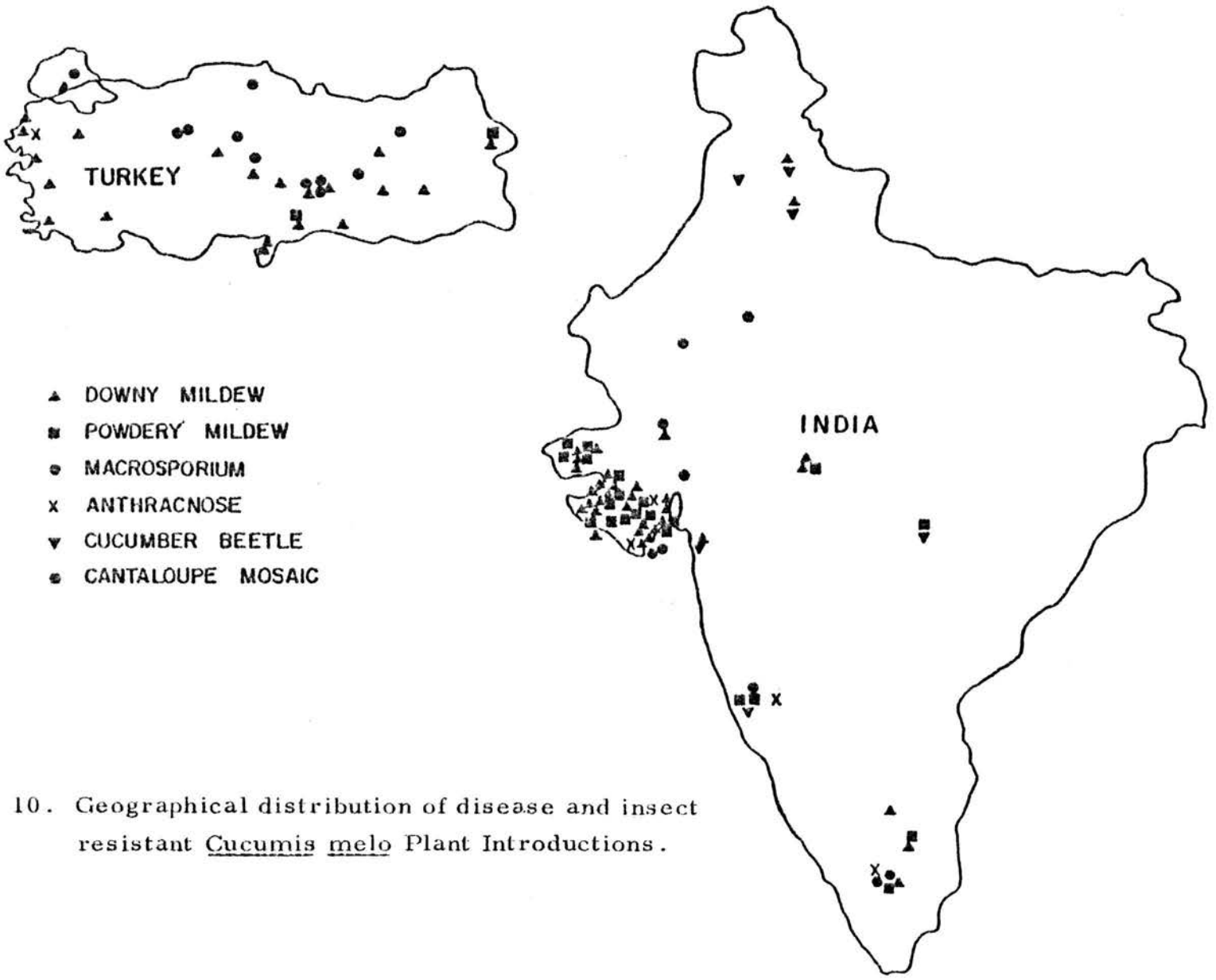

Figure 10. Geographical distribution of disease and insect resistant Cucumis melo Plant Introductions.

resistance or tolerance to cantaloupe mosaic in collections from China and Korea, while Chambliss and Cuthbeth (1968) screened PI's and varieties for resistance to the cucumber beetle and found resistance mostly in Indian accessions (Figure 10).

Isoperoxidase genotype and gene frequencies of resistant C. melo accessions were calculated (Table 4). The overall frequency of the Px_{2A} allele appeared to be 0.53, but a surprisingly high gene frequency was detected among PI's with resistance to gummy stem blight, anthracnose, cucumber beetle and cantaloupe mosaic. Peroxidases have been related to disease resistance and these data suggest that C. melo plants with the Px_{2A} peroxidase cluster have more resistance to various diseases and insects (except mildews and Macrosporium) than plants with the $Px_{2B}Px_{2B}$ genotype.

Host-parasite relationships, used to locate gene centers of cultivated plant species (Leppik, 1966), point to India as the area of diversity of this large and polymorphic Cucumis species, since most sources of disease and insect resistance occur among Indian accessions. Linguistic data from Filov et al. (1973) support this conclusion. If India is accepted as the area of diversity of this species, the populations of Turkey, Syria and China can be considered as more peripheral. While Mayr's observations indicated that the degree of phenotypic polymorphism decreases towards the border of the species range, electrophoretic studies showed that marginal populations were as heterozygous as central ones (Lewontin, 1974). More marginal

Table 4. Peroxidase genotype and gene frequencies of Plant Introductions of Cucumis melo resistant to various diseases and insects.

Disease or Insect	Peroxidase Genotype Frequencies			N	Gene Frequency	X^2
	$Px_{2A}Px_{2A}$	$Px_{2A}Px_{2B}$	$Px_{2B}Px_{2B}$		$P_{Px_{2A}}$	
downy mildew	.18	.56	.26	72	.46	1.0
powdery mildew	.29	.42	.29	24	.50	0
gummy stem blight	.50	.50		2	.75	1.0
anthracnose	.43	.57		7	.71	2.6
Macrosporium	.28	.39	.33	18	.47	0.1
cucumber beetle	.59	.41		17	.80	11.8**
cantaloupe mosaic	.50	.50		6	.75	3.0

**Significant at the .01 level of probability.

C. melo populations had a high degree of homozygosity for the Px_{2B} peroxidase allele, but the geographic variation in peroxidase isozyme pattern could not be correlated with the variation in morphological characters.

The neutralist theory predicts that the peroxidase alleles are truly isoallelic and that the changes in allele frequency are the result of stochastic processes (Kimura and Ohta, 1971). Establishment of a new population from a few plants (Founder principle, Mayr, 1970) may have caused the low Px_{2A} gene frequency in some populations but would have been associated with a higher degree of homozygosity for genes governing fruit shape, taste and color. A nonrandom sampling of Plant Introductions, collected mainly from market places, does not appear likely since widely separated populations have the same peroxidase allele frequencies.

The selectionist theory predicts that slightly different selection pressures are responsible for the observed distribution of peroxidase alleles (Lewontin, 1974). The Px_2 alleles differ in electrophoretic mobility, which may be associated with somewhat different kinetic properties and result in slightly different selection coefficients. The nonrandom distribution of the peroxidase allele frequency seems to support this hypothesis, but additional research is needed to better understand the evolutionary behavior of this Cucumis species.

C. prophetarum, PI 179922, from India has 2n=24 chromosomes (Dane and Tsuchiya, 1976), the Px_1 and Px_{2A} peroxidases,

morphological characteristics like some of the non-cultivated C. melo accessions and is cross-incompatible with other C. prophetarum PI's. The data suggest that PI 179922 belongs to Cucumis melo.

The origin of this cultivated Cucumis species and its relationship to the other cultivated and wild Cucumis species will be discussed in the "Relationship Between the Wild and Cultivated Cucumis Species" section of this chapter.

Cucumber (Cucumis sativus)

Cucumber Plant Introductions from all over the world and known U.S. varieties were found to have an identical peroxidase banding pattern. The Px_1 peroxidase, present in other cultivated and most of the wild Cucumis species, is absent, but a cluster of three bands, b, c and d, at the Px_2 locus are always present (Figure 11).

The peroxidase banding pattern of wild species, Cucumis hardwickii (PI 215589) and Cucumis trigonus (PI 215589 and 271337) is identical to that of C. sativus, their chromosome number is 2n=14 (Dane and Tsuchiya, 1976) like the cucumber, which indicates that these species are nothing more than cucumber varieties escaped from cultivation rather than the ancestors of the cucumber as proposed by de Candolle (1882). Deakin et al. (1971) reached similar conclusions about C. hardwickii based on hybridization results of this species with Cucumis sativus.

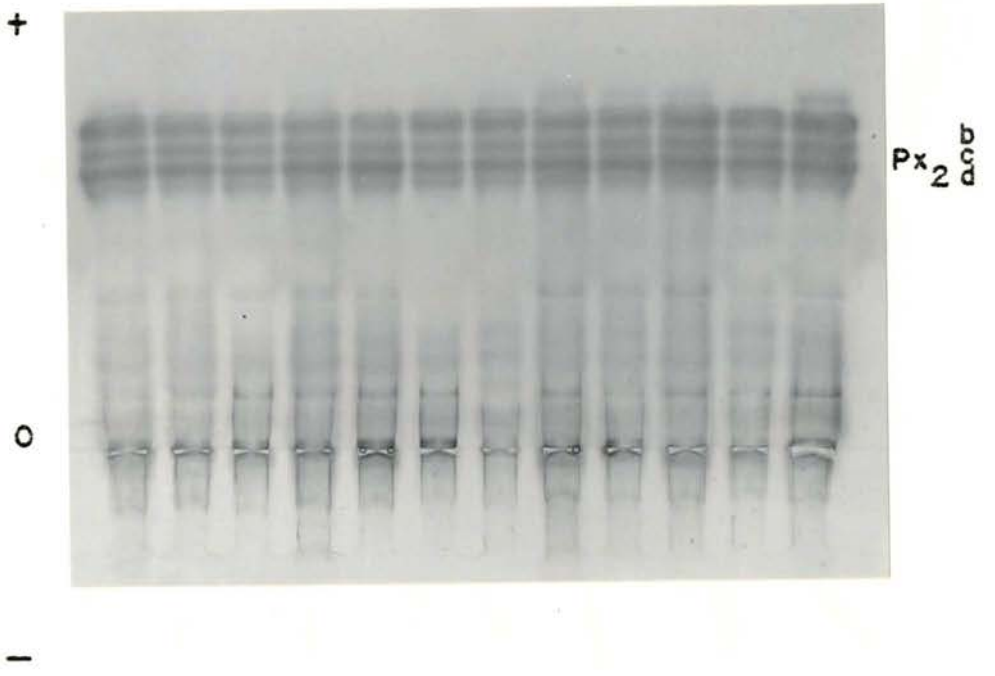

Figure 11. Peroxidase banding pattern of Cucumis sativus Plant Introductions.

The relationship of this Cucumis species with other cultivated and wild Cucumis species will be discussed in the "Relationship Between the Wild and Cultivated Cucumis Species" section of this chapter.

West India Gherkin (Cucumis anguria)

The West India or Bur gherkin, known in cultivated and semi-wild state in the Americas, is thought to have originated from a wild African species, C. longipes. The cultivated and wild forms were found to be conspecific and retained as variety anguria or longipes of C. anguria, respectively (Meeuse, 1958).

Ten Plant Introductions collected in Brazil, Ethiopia, Rhodesia, South and South West Africa, and classified as C. anguria, C. ficifolius and C. longipes were obtained from the U.S.D.A., A.R.S., PI Service (Table 1). These monoecious, annual and diploid (2n=24, Dane and Tsuchiya, 1976) accessions can be divided into three morphologically distinct groups (Table 5):

1. The Brazilian (PI 147065 and 196477) and Ethiopian (PI 233646 and 320052) accessions are characterized by the presence of five-lobed leaves, oblong fruits and ovaries and a distinct plant development pattern resulting in the formation of a single erect stem of approximately 20 cm followed by the development of prostrate stems branching off near the base of the stem and attaining a length of 1-2 m. Differences do exist in ovary and fruit morphology, however. While

Table 5. Source and morphological characteristics of Cucumis anguria Plant Introductions.

PI Number	Source	Leaf Morphology	Ovary Morphology in cm	Fruit Morphology in cm	Seed Size in cm
147065	Brazil	5-lobed rounded	oblong, 1.2 x 0.5 0.2 cm spines, p=2.8* cm	oblong, 4.7 x 2.9 0.1 - 0.8 cm spines p=6.4 cm	.51 x .23 x .11
196477	Brazil	5-lobed rounded	oblong and spiny		.51 x .24 x .11
233646	Ethiopia	5-lobed rounded	oblong, 1.1 x 0.5 0.2 cm hairs p=4.2 cm	oblong, 4.7 x 3.2 0.2 cm hairs p=6.3 cm	.50 x .22 x .11
320052	Ethiopia	5-lobed rounded	oblong, 1.2 x 0.6 0.2 cm hairs p=5.9 cm		.50 x .23 x .11
249894	S. Rhodesia	slightly 3-lobed	round and spiny	globular, 2.4 x 1.8 0.5 - 0.8 cm spines p=1.7 cm	.58 x .26 x .13
282442	S. Rhodesia	slightly 3-lobed	round and spiny	globular, 3.9 x 3.3 .6 - 1.2 cm spines p=3.4 cm	.55 x .25 x .12
249895	S. Rhodesia	5-lobed lobulate	globular 0.8 x 0.5, 0.2 cm spines, p=6.0 cm	globular, 4.5 x 3.4 1.5 cm spines (6/cm^2) p=10 cm	.55 x .24 x .13

Table 5. Continued.

PI Number	Source	Leaf Morphology	Ovary Morphology in cm	Fruit Morphology in cm	Seed Size in cm
249896	N. Rhodesia	5-lobed pointed	globular and spiny	globular, 2.9 x 2.6 0.1 - 1.5 cm spines ($4/cm^2$), p=9.5 cm	.58 x .26 x .13
249897	S.W. Africa	3-5 lobed	globular, spiny		.58 x .27 x .14
364475	S. Africa	5-lobed lobulate	glubular and spiny	globular, 3.2 x 2.7 .5 - .8 cm spines p=7.7 cm	.51 x .23 x .12

* p = peduncle length.

the Plant Introductions from Brazil develop ovaries with 0.2 cm long spines and fruits with thick curled spines of 0.1-0.8 cm, the Ethiopian accessions form hairy fruits and ovaries (Table 5, Figure 12c).

2. The accessions from S. Rhodesia (PI 249894 and 282442) are distinguishable from the other PI's by their slightly three-lobed leaves and globular and spinu fruits and ovaries (Figure 12a).

3. The other African PI's appear as a morphological mixture of PI 147065, 196477, 233646, 320052 (group 1) and 249894, and 282442 (group 2). Their leaves are five-lobed but more lobulate and pointed than the leaves of the Ethiopian and Brazilian accessions and their fruits and ovaries are globular and spiny. PI 249896 forms approximately four spines per cm^2 fruit surface, while all other spiny fruited South African accessions develop 7-8 spines per cm^2 fruit surface (Table 5, Figure 12b).

Morphologically different plants hybridize quite easily following hand pollination (Table 6) and produce vigorous and fertile F_1's with 98% or more normal and three-porate pollen (Table 7), thus confirming results of Meeuse (1958) and Deakin et al. (1971) that the different C. anguria Plant Introductions are varieties of a single species.

The morphologically distinct C. anguria plants can also be distinguished by their peroxidase banding patterns. All PI's have Px_1, a Px_2 cluster and peroxidases at the Px_3 locus (Figure 13). The Px_2 locus lacks the clear multiple band definition of C. sativus or C. melo plants but shows a similar migrational behavior. Only PI364475

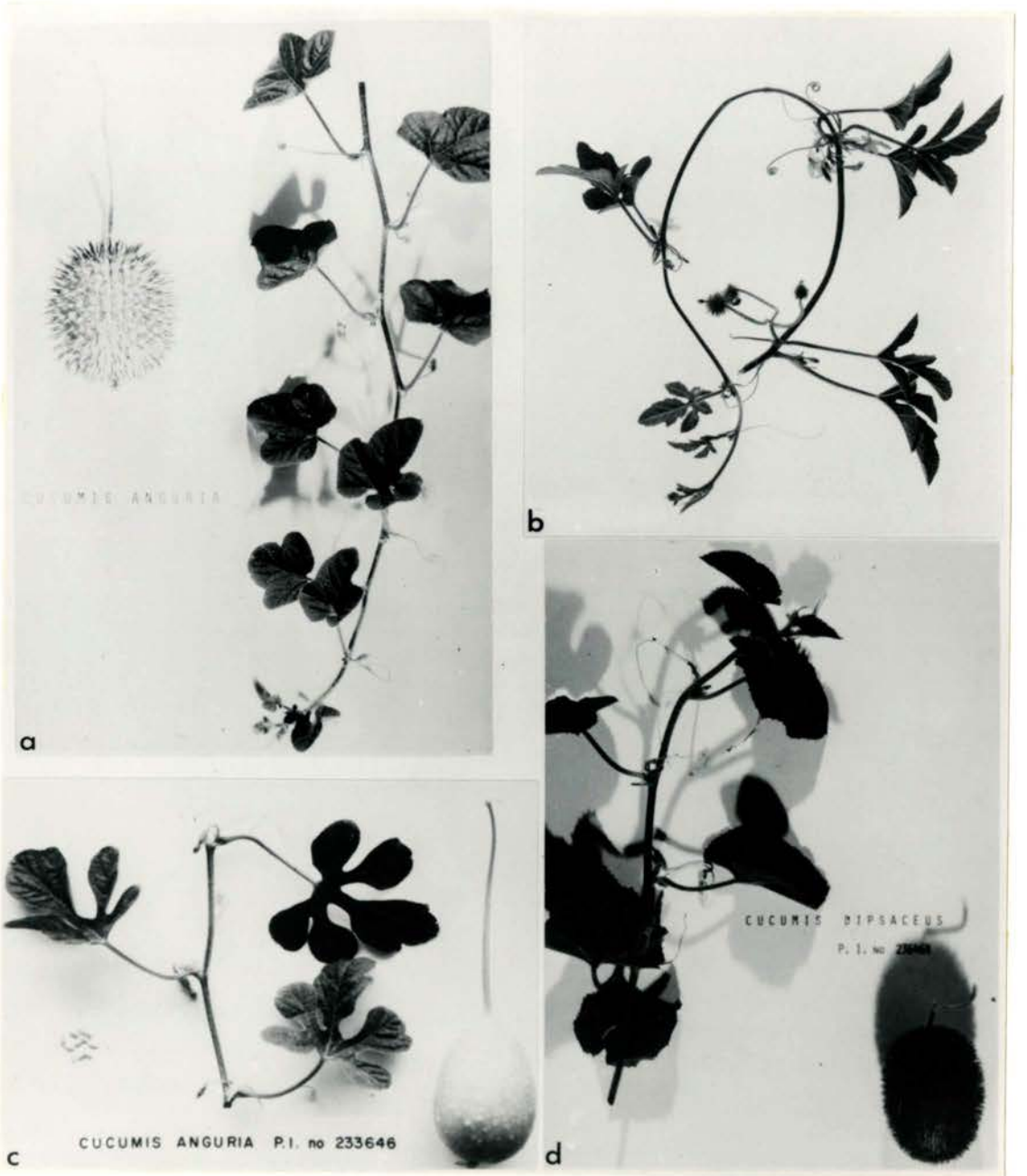

Figure 12. a. C. anguria var. longipes (PI 282442); b. C. anguria var. longipes (PI 364475); c. C. anguria var. anguria (PI 233646); and d. C. dipsaceus.

Table 6. Pollen morphology and fertility of Cucumis anguria PI's and intraspecific F_1 plants.

PI of F_1	Percent Pollen		Percent Pollen	
	3-porate	4-porate	normal	aborted
233646	100	0		
364475	100	0		
147065 x 282442	98	2	100	0
282442 x 364475	100	0	98	2

Table 7. Hybridization studies of Cucumis anguria Plant Introductions.

Female Parent	Male Parent*					
	147065 196477	233646 320052	249896	249894 282442	249895	364475
147065 196477				F		
233646 320052						
249896	+	+		F		
249894 282442			+			F
249895				+		+
364475						

*F = F_1 seedlings. + = fruit and seed formation.

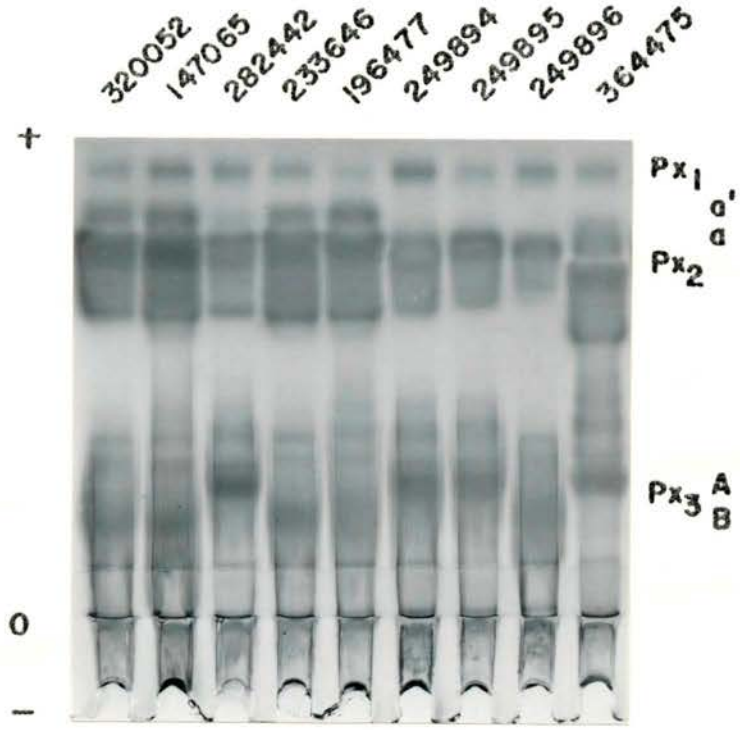

Figure 13. Peroxidase banding pattern of Cucumis anguria PI's.

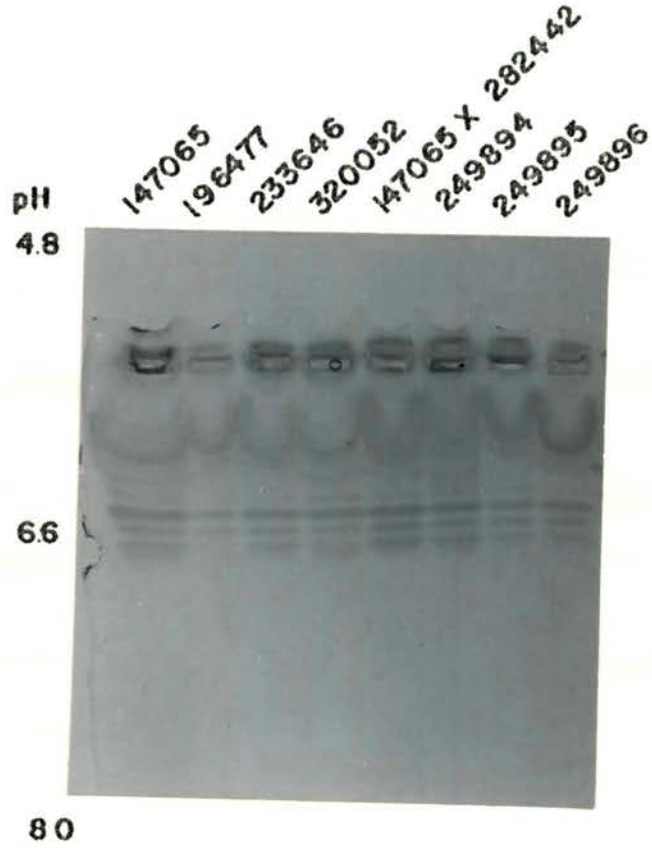

Figure 14. Peroxidase isoelectric focusing pattern of C. anguria PI's.

has a Px_{2B} cluster, while all other C. anguria accessions have a cluster of peroxidase bands with a migrational behavior similar to that of the Px_{2A} cluster of C. melo plants. Polymorphism at the Px_3 locus is apparent. While PI 147065, 196477, 233646, 320052 and 249896 have Px_{3B}, PI 282442, 249894, 249895 and 364475 have Px_{3A} and PI 249897 shows both Px_{3A} and Px_{3B} peroxidases (Figure 13). F_1 plants of (147065 x 282442) and (249896 x 282442) contain Px_{3A} and Px_{3B} peroxidases, which is indicative of the codominant nature of the peroxidases at the Px_3 locus. Genetic studies with F_2 seedlings were carried out but failed to show Px_3 band definition presumably because the Px_3 peroxidases are expressed at a relatively late developmental stage. If o-dianisidine is used as the peroxidase substrate instead of o-tolidine, PI 147065, 196477, 233646, 320052 and 249896 show an additional Px_{3C} band, while PI 249894, 282442, 249895 and 364475 have an additional Px_{3B} band and F_1 plants of (147065 x 282442) show all three Px_3 (A, B and C) peroxidases in their leaf and stem tissues. Whether the additional band in the o-dianisidine peroxidase banding pattern is an artifact or not, remains to be investigated.

Not only do the C. anguria accessions show a distinctive peroxidase banding pattern upon electrophoresis but also upon isoelectric focusing. Figure 14 shows the identity of the peroxidase band distributions upon isoelectric focusing of plant juice extracted from different C. anguria plants. Intraspecific variation in α-esterases or

glutamate oxaloacetate transaminases could not be detected. All these results support the conclusion that the C. anguria accessions are conspecific.

Although Meeuse (1958) considered strain "Clarke 156" (PI 249896) and strain "Gatooma 26" (PI 282442) as Cucumis anguria variety longipes and the ancestors of the cultivated gherkin, C. anguria var. anguria; the morphological, hybridization and zymogram studies of these and other C. anguria accessions indicate that:

1. Not only do PI 147065, 196477, 233646, 320052 but also does PI 249896 belong to variety anguria since the Ethiopian and Brazilian accessions have similar morphological characteristics and all contain the Px_{3B} peroxidase in their leaf and stem tissues.

2. The cultivated gherkin, PI 147065 and 196477 of Brazil, descended from accessions 233646 or 320052 of Ethiopia.

3. PI 249894, 249895, 282442 and 364475 are members of C. anguria var. longipes, characterized by the presence of peroxidase band Px_{3A}.

4. PI 249897, which is morphologically similar to F_1 plants of (249896 x 282442), and contains both Px_{3A} and Px_{3B} in its leaf and stem tissues can be considered as a cross between PI 249896 and 282442.

The five-lobed leaf condition is dominant over the slightly three-lobed leaf condition (Figure 12b) since F_1 plants of (147065 x 282442) and (282442 x 364475) develop five-lobed leaves. Also, the globular,

heavily spined ovary and fruit character is dominant over all others since F_1 plants of (147065 x 282442) and (249896 x 282442) develop globular, heavily spined fruits and ovaries. This appears to indicate that PI 282442 and 249894 (group 2) descended from PI 249895 and 364475 and that homozygosity of a recessive mutation led to the slightly three-lobed leaf condition characteristic of these accessions.

The relationship of C. anguria with the wild and cultivated Cucumis species will be discussed in the "Relationship Between the Wild and Cultivated Cucumis Species" section of this chapter.

Cucumis Species from Ethiopia

The distribution of Cucumis accessions indigenous to Ethiopia is outlined in Figure 15. The U.S.D.A., A.R.S., Plant Introduction Service classified PI 193498 and 236468 as C. dipsaceus, PI 280231 and 273649 as C. pustulatus, PI 193967 and 273648 as C. propheta-rum, PI 196844, 233646 and 273648 as C. ficifolius, and PI 233646 and 320052 as C. anguria. Jeffrey (1967), however, listed only three species, C. dipsaceus, C. ficifolius, and C. aculeatus as being native to Ethiopia in his flora of tropical East Africa. Morphological studies of the accessions (Table 8) indicated that PI 280231 follows Jeffrey's description of C. ficifolius and that PI 193967, 196844, 273648 and 273650 are morphologically similar and follow Jeffrey's description of C. aculeatus. All Ethiopian accessions are monoecious, but, while C. anguria var. anguria and C. dipsaceus are annual, C. ficifolius

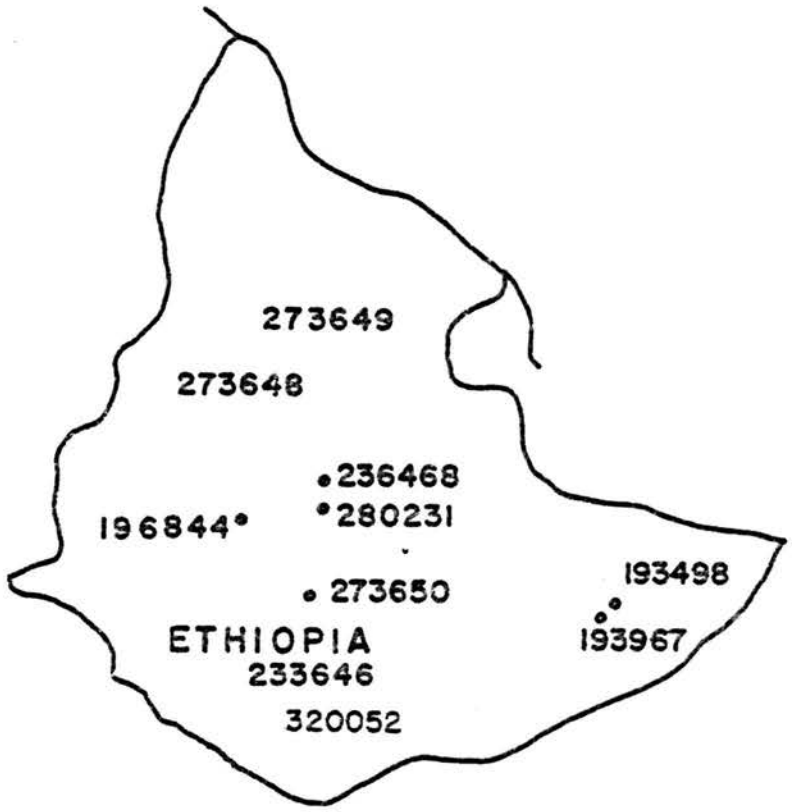

Figure 15. Geographical distribution of Ethiopian Cucumis species.

Table 8. Morphological characteristics and chromosome number of Cucumis Plant Introductions from Ethiopia.

PI Number	Species	2n	Leaf Morphology	Ovary Morphology	Fruit Morphology	Seed Size in cm
193498 236468	C. dipsaceus	24	ovate non-lobed	ellipsoid, 1.2 x 0.6 cm, 0.2 - 0.3 cm spines, p< 0.5* cm	ellipsoid, 3.9 x 2.9 cm, densely covered with 0.5-0.6 cm spines	.50 x .22 x .09
280231	C. ficifolius	24	3-5 lobed hairy stems	1.0 x 0.5 cm, 0.2 cm spines, p=2.5	globular, 3.6 x 2.8 cm, p=1.6 cm	.56 x .29 x .15
193967	C. aculeatus	48	3-5 lobed, prickly leaves and stems	0.9 x 0.5 cm with 0.2-0.3 cm spines p=2.1 cm	3.8 x 2.9 cm p=1.5 cm	.49 x .25 x .12
196844	C. aculeatus	48	3-5 lobed prickly	1.4 x 0.6 cm, 0.2 cm spines, p=2.4cm	3.8 x 3.0 cm p=2.2 cm	.56 x .27 x .13
273648	C. aculeatus	48	3-5 lobed prickly	1.2 x 0.7 cm, 0.2 cm spines, p=2.8cm	4.3 x 3.7 cm p=2.2 cm	.58 x .28 x .14
273649	C. aculeatus	48	3-5 lobed prickly		4.1 x 2.9 cm	.62 x .29 x .15
273650	C. aculeatus	48	3-5 lobed prickly		4.3 x 2.9 cm .05 cm spines p=2.3 cm	.53 x .26 x .13

*p=peduncle length.

and C. aculeatus are perennial herbs. Chromosome studies indicated that all Ethiopian species are diploid with 2n=24 chromosomes, except C. aculeatus (PI 193967, 196844, 273648, 273649 and 273650) which is tetraploid with 2n=48 chromosomes (Table 8, Dane and Tsuchiya, 1976).

Cucumis dipsaceus

The C. dipsaceus accessions are morphologically similar, have tricolporate pollen and are distinguishable from other Cucumis species by their nonlobed leaves and heavily spined fruits and ovaries (Table 8, Figure 12d). Their stem and leaf tissues show the Px_1, a cluster of four (a, b, c and d) Px_2, Px_2a', and the Px_{3B} peroxidase isozymes (Figure 16, Table 36). Individual C. dipsaceus plants (12) and the offspring of selfed plants displayed that same peroxidase banding pattern and indicated homozygosity of the Cucumis dipsaceus peroxidase loci. The presence of Px_2a' (with a frequency of 0.7) is thought to be dependent on the developmental stage of the plants.

Cucumis ficifolius

This perennial diploid is morphologically similar to C. aculeatus but while C. aculeatus' stems and leaves are prickly, C. ficifolius is hairy (Table 8). Both species have an efficient vegetative reproductive system in that rooting occurs frequently at nodes. C. ficifolius pollen mother cells showed normal bivalent chromosome pairing (24 PMC's with 12 II, 2 PMC's with 11 II and 2 I) and produce

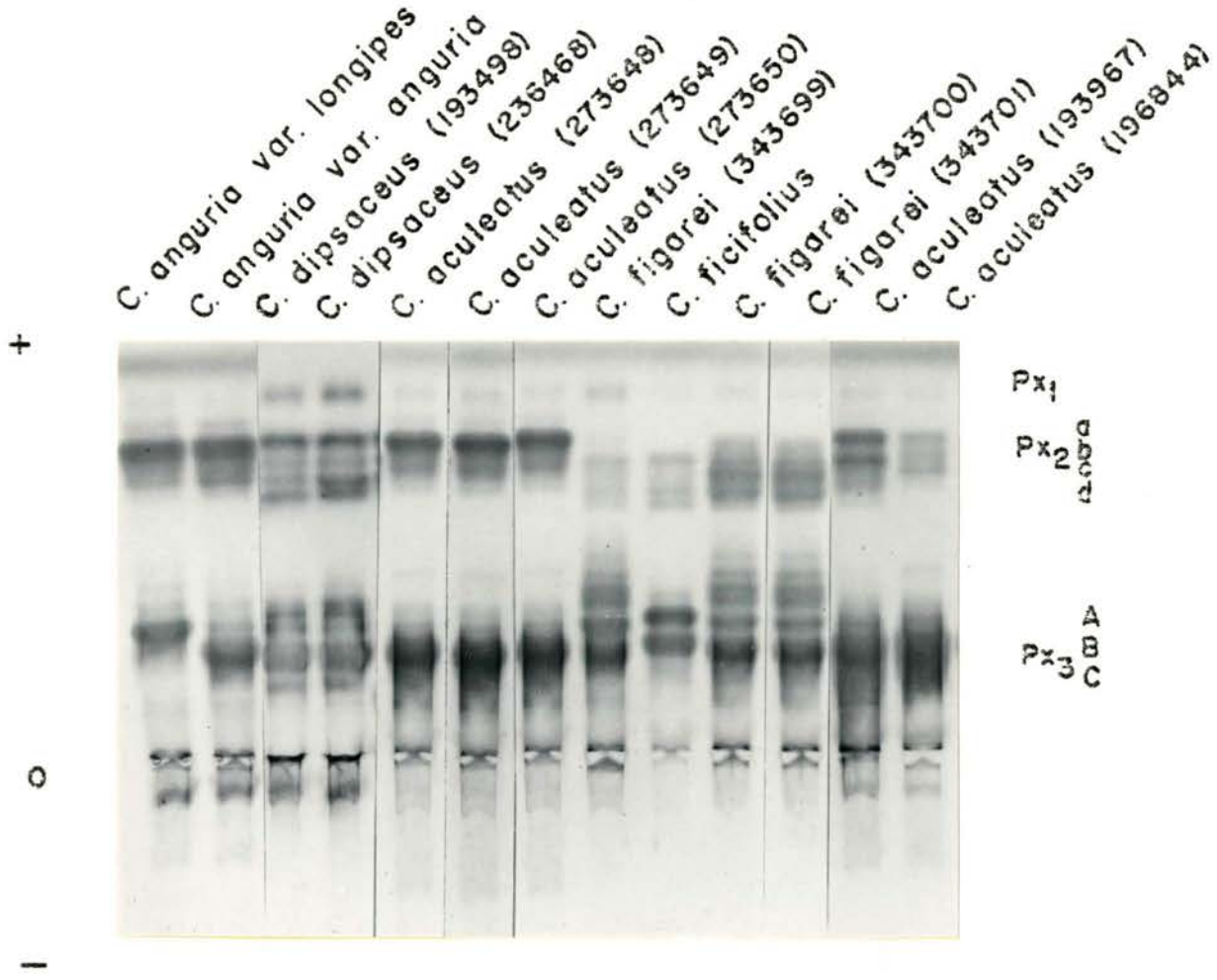

Figure 16. Peroxidase banding pattern of Cucumis aculeatus, C. anguria, C. dipsaceus, C. figarei and C. ficifolius.

pollen with three pores. Figure 16 shows the peroxidase banding pattern of the stem and leaf tissues of C. ficifolius. Px_1, a cluster of four (b, c, d and d') Px_2 bands, Px_{3B} and sometimes Px_{3A} peroxidases are found (Table 36).

Cucumis aculeatus

Meiotic studies of this tetraploid Cucumis species (PI 273648) showed the presence of 24 II in 16 metaphase cells and 13 diakinesis cells. Tetraploidy was not accompanied by an increase in the number of pores per pollen grain or the development of abnormal or aborted pollen (Table 9). The presence of bivalents at metaphase I and lack of four or more porate pollen grains is indicative of the allotetraploid nature of this Cucumis species.

The C. aculeatus accessions are morphologically similar (Table 8) and show a high degree of intraspecific cross-compatibility (Table 10). PI 273648 differs slightly in leaf and fruit morphology from the other accessions. Pollen mother cells of (273650 x 273648) F_1 plants showed a reduction in the formation of bivalent chromosome configurations (1 PMC with 19 II and 10 I, 1 PMC with 16 II and 16 I). Whether this is caused by chromosomal differences between PI 273648 and the other accessions or is common to intraspecific C. aculeatus F_1's remains to be investigated.

Similarity of the C. aculeatus accessions was also demonstrated by their peroxidase banding patterns. Px_1, a cluster of Px_2 bands at

Table 9. Pollen morphology and fertility of C. aculeatus accessions.

	Percent Pollen		Percent Pollen	
	3-porate	4-porate	normal	aborted
PI 193967	96	4	96	4
PI 273648	99	1	99	1
PI 273649	99	1		

Table 10. Hybridization studies of C. aculeatus Plant Introductions.

	Male Parent*				
Female Parent	193967	196844	273648	273649	273650
193697		+		+	+
196844	+		+	F	+
273648	+	+		+	
273649	+	+	-		+
273650		+	F		

*+ indicates fruit and seed formation.
F indicates the formation of vigorous F_1 plants.
- indicates the lack of fruit development upon cross-pollination.

the same migrational distance as the Px_{2A} and Px_{2B} clusters of C. melo, Px_{3B} and sometimes Px_{3A} peroxidase isozymes (Figure 16, Table 36) were present in their leaf and stem tissues. The peroxidase banding patterns of ten (273650)-S_1 plants were identical and showed Px_1, Px_2 bands a, b, c and d, and Px_{3B}. The low peroxidase band frequencies of Px_2 a' and d', and Px_{3A} peroxidases must be attributed to ontogenetic or developmental effects because of homozygosity of the Px_1, Px_2 (a, b, c, d), Px_{3B} C. aculeatus peroxidase pattern.

The peroxidase banding patterns obtained after isoelectric focusing of the plant juice of the tetraploid accessions (Figure 17) also showed identity. Glutamate oxaloacetate transaminase (GOT) isozyme patterns of the accessions (PI 273648 and 196844) were identical, while the esterase patterns of PI 273648, 196844 and 273649 showed differences but lacked reproducibility.

The Relationship Between the Ethiopian Cucumis Species

Hybridization studies between the Cucumis species from Ethiopia showed a high degree of interspecific cross-compatibility and resulted in the production of vigorous interspecific F_1 plants (Table 11). Their fertility was low, however. The diploid C. anguria var. anguria (233646) x C. dipsaceus F_1 formed many 4-porate (13%) and abnormal (87%) pollen. Triploid F_1's showed a reduction in the formation of staminate flowers and in pollen fertility because of

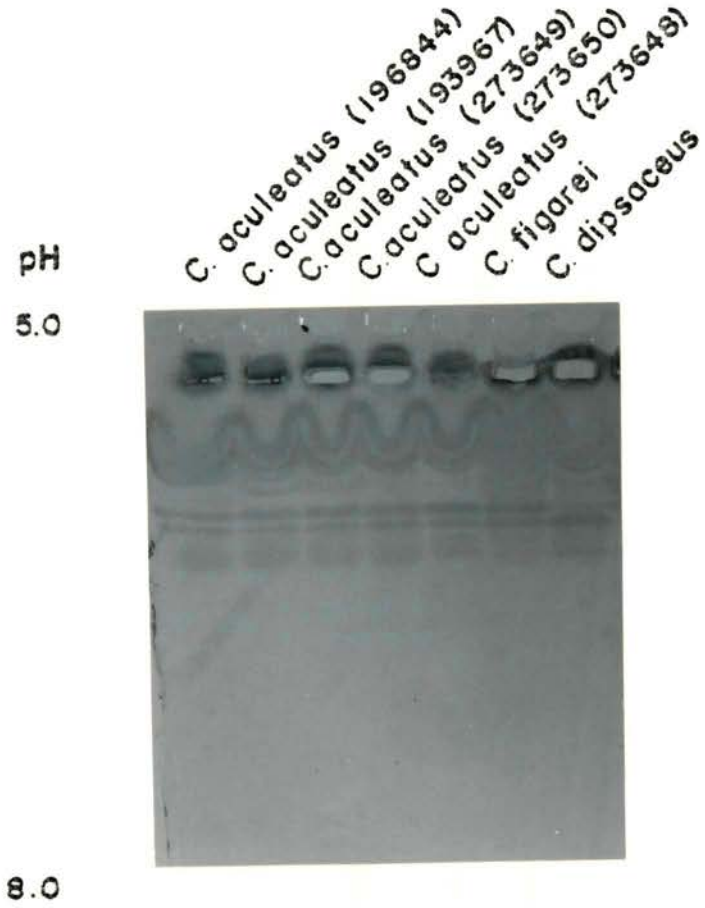

Figure 17. Peroxidase isoelectric focusing pattern of C. aculeatus, C. dipsaceus, and C. figarei.

Table 11. Hybridization studies of Cucumis species from Ethiopia.

Female Parent	Male Parent* 1	2	3	4
1. C. aculeatus		F	F	F
2. C. anguria var. anguria			F	
3. C. dipsaceus				
4. C. ficifolius	+	F	F	

*F indicates the formation of vigorous F_1 plants.
+ indicates the production of fruit and seed upon cross-pollination.

Table 12. Meiotic chromosome configurations at diakinesis and metaphase I in F_1 plants of C. aculeatus (PI 273650) x C. ficifolius (PI 280231).

Chromosome Configurations III	II	I	Number of Pollen Mother Cells
	12	12	32
1	11	11	4
	11	14	4
0.1 III	11.8 II	12.1 I	$\bar{x}$

irregular meiotic chromosome separations. (C. aculeatus (273650) x C. ficifolius) F_1 plants produced mainly 3-porate (96%; 4% 4-porate) and many aborted (25%; 75% normal) pollen.

Meiotic chromosome studies of interspecific F_1 plants showed an average of 11.8 II and 0.1 III in pollen mother cells of C. aculeatus (PI 273650) x C. ficifolius (Table 12, Figure 18a); 10.2 II and 0.3 III in PMC's of C. aculeatus (PI 273648) x C. anguria (PI 233646) (Table 13, Figure 18d); and 10.3 II in PMC's of C. anguria var. anguria x C. dipsaceus F_1's (Table 14). The high frequency of bivalent chromosome pairing and very limited trivalent formation indicate a high level of chromosome homology between the genomes of the diploid species and one genome of the allotetraploid C. aculeatus.

The Ethiopian species do not only show similar peroxidase banding patterns (Figure 16, Table 36) and identical GOT isozyme patterns (Figure 35), but also similar chromatographic flavonoid patterns (Brown et al., 1969). These electrophoretic, other isoelectric focusing (Figures 33 and 34) and meiotic chromosome studies indicate that the species are very closely related and must have evolved from the same ancestor.

Cucumis Species from Nigeria

One Cucumis species, classified by the U.S.D.A., A.R.S., PI Service as C. pustulatus (PI 343699, 343700, 343701) was collected in Nigeria. The plants are morphologically identical, develop

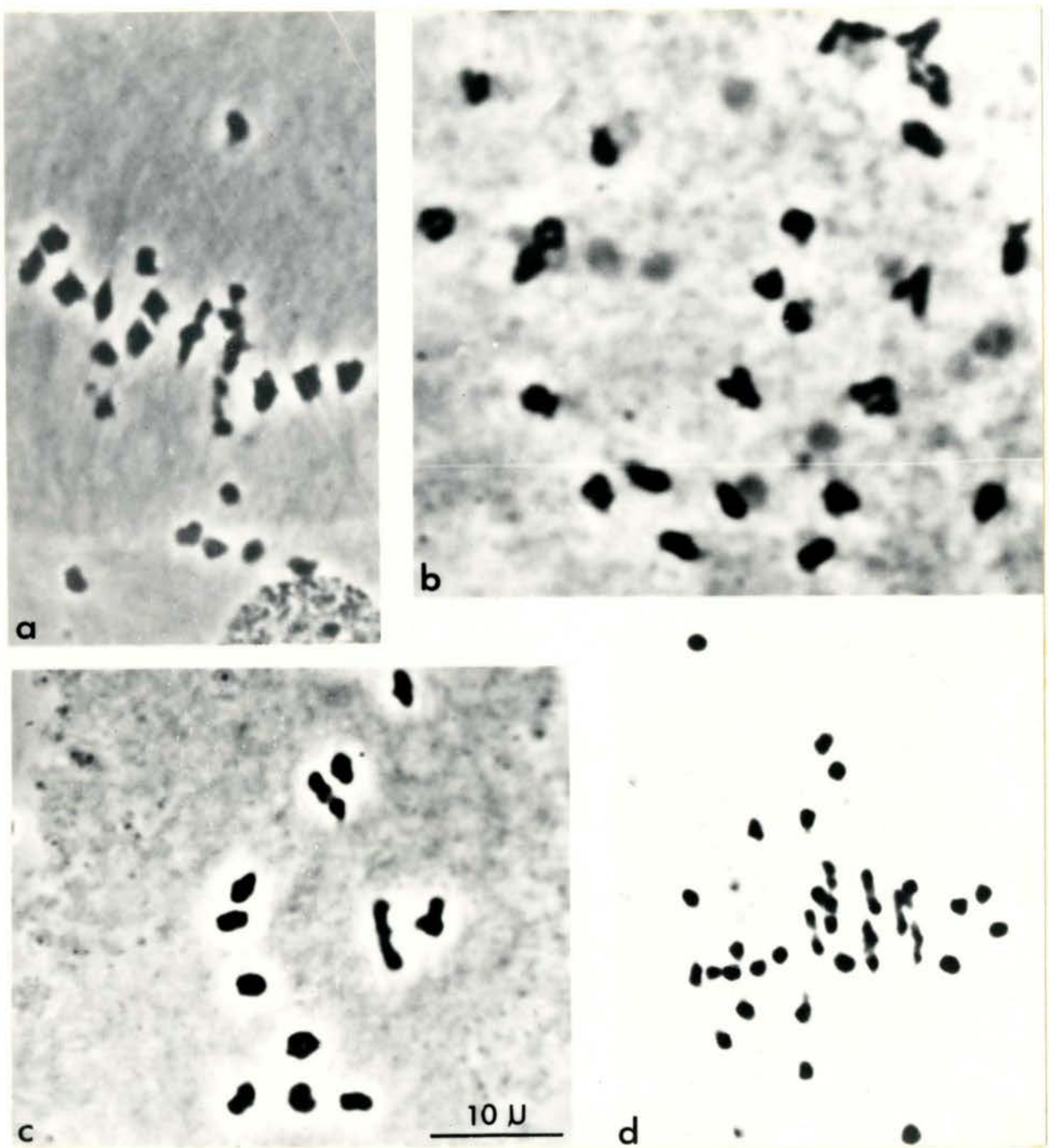

Figure 18. Meiotic chromosome configurations of (a) triploid C. aculeatus x C. ficifolius with 12 II + 12 I, (b) tetraploid C. aculeatus with 24 II, (c) diploid C. ficifolius with 12 II, and (d) triploid C. aculeatus x C. anguria with 8 II + 20 I.

Table 13. Meiotic chromosome configurations at diakinesis and metaphase I in F_1 plants of C. aculeatus (PI 273648) x C. anguria (PI 233646).

Chromosome Configurations			Number of
III	II	I	Pollen Mother Cells
	12	12	4
1	11	11	1
	11	14	1
1	10	13	1
	10	16	3
2	8	14	1
1	9	15	1
	9	18	1
	8	20	3
	7	22	1
1	6	21	1
0.3 III	10.2 II	15.6 I	$\bar{x}$

Table 14. Meiotic chromosome configurations at metaphase I in F_1 plants of C. anguria (PI 233646) x C. dipsaceus.

Chromosome Configurations		Number of
II	I	Pollen Mother Cells
12		3
11	2	4
10	4	8
9	6	3
8	8	1
10.3 II	3.5 I	$\bar{x}$

slightly 3-lobed leaves, ovaries of 1.2 x 0.6 cm with bulbous spines, fruits of 6.1 x 4.5 cm with scattered pustules and a 9 cm long stalk, and seed of .61 x .29 x .14 cm (Figure 25). This monoecious and perennial species corresponds with Jeffrey's description and distribution range (1967) of C. figarei.

Chromosome studies showed 2n=72 chromosomes in somatic cells and 36 II in pollen mother cells (12) (Figure 19), although more cells need to be analyzed in order to determine the hexaploid nature of this species since quadrivalent chromosome associations may be present (see "Relationship Between the Wild and Cultivated Cucumis Species" in this chapter). The increase in ploidy level was associated with an increase in the number of pores per pollen grain. Seventy-four percent of the staminate flowers of this species developed 3-porate pollen, 26% four or more porate pollen.

The peroxidase isozymes present in the stem and leaf tissues of C. figarei accessions are shown in Figure 16. Px_1, a cluster of 4-6 Px_2 bands, Px_{3A} and Px_{3B} and additionally slowly migrating peroxidases in the 8% gel layer are present (Table 36). The species' relationship with the other Cucumis species will be discussed in the "Relationship Between the Wild and Cultivated Cucumis Species" section of this chapter.

Figure 19. Meiotic chromosome configurations at metaphase I in the hexaploid Cucumis figarei (PI 343699) showing 36 II, 3000x.

Cucumis Species from South and South West Africa

The collection sites of Cucumis Plant Introductions of Southern Africa are outlined in Figure 20. Although Leppik (1966) considers East Africa as the primary gene center of the genus, most of the Cucumis species are indigenous to Southern Africa and have adapted to widely different environments from rock crevices (C. asper) to soils with deep fine sand (C. humifructus) and have differentiated into distinctly different morphologies.

Cucumis africanus, C. leptodermis, and C. myriocarpus

These three South African species are characterized by their annual growth habit, monoecious sex expression, relatively small ovaries and fruits, 2n=24 chromosomes (Dane and Tsuchiya, 1976), and the presence of cucurbitacin A in their fruits (Rehm, 1960). Meeuse (1962) considers C. leptodermis not more than a variety or subspecies of C. myriocarpus since they hybridize quite easily not only after artificial cross-pollination but also in nature.

The U.S.D.A., A.R.S., P.I. Service classified PI 203977, 282449, 299568 and 374153 as C. myriocarpus, PI 282447 as C. leptodermis, PI 374151 as C. africanus, PI 299568 also as C. zeyheri and PI 275571 as C. hookeri. Their morphological characteristics, chromosome number and classification according to Meeuse (1962) are presented in Table 15.

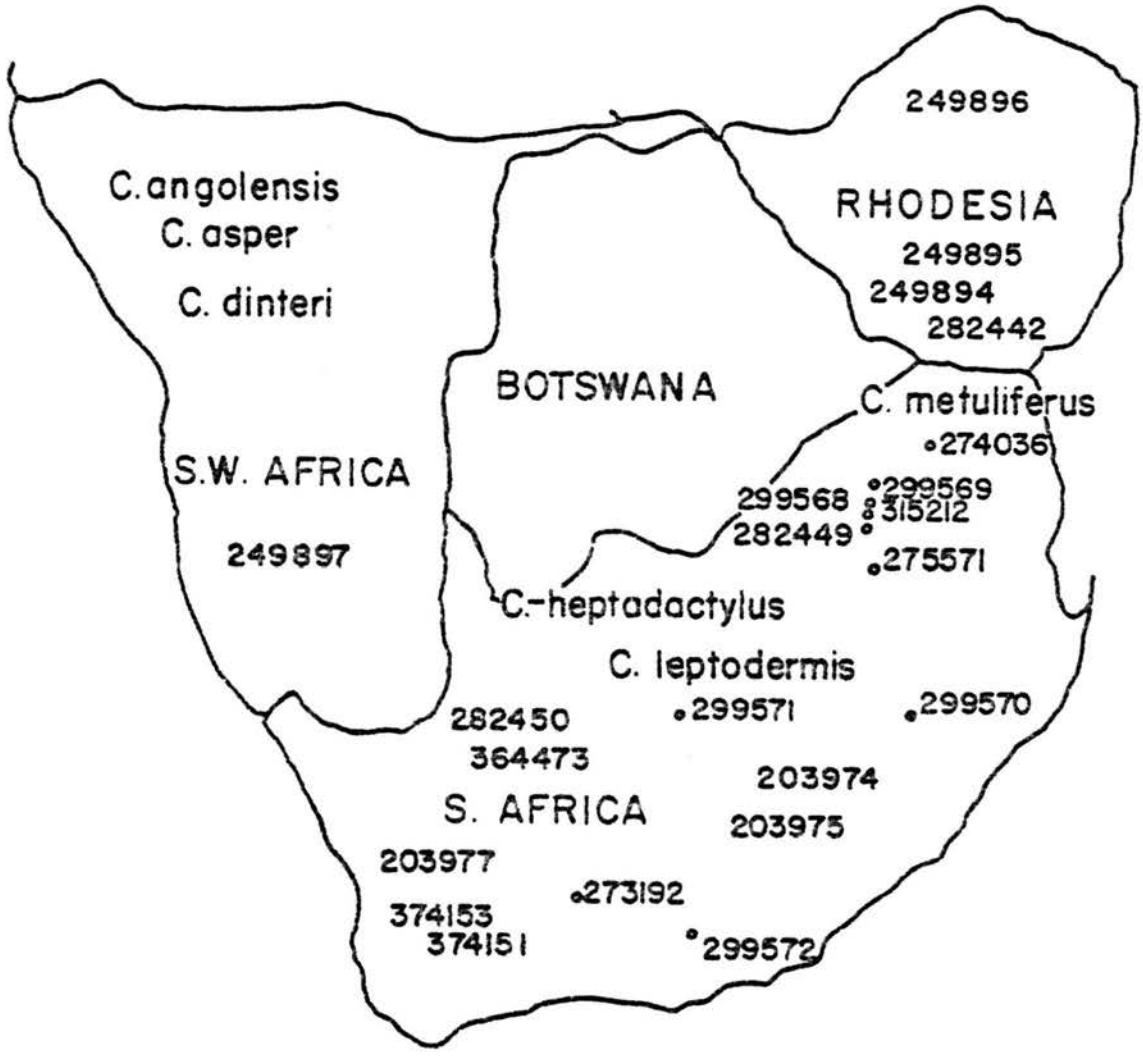

Figure 20. Geographical distribution of Cucumis species from Southern Africa.

Table 15. Morphological characteristics and chromosome number of Cucumis africanus, C. leptodermis and C. myriocarpus from South Africa.

PI Number	Species	2n	Leaf Morphology	Ovary Morphology	Fruit Morphology	Seed Size in cm
203975	C. africanus	24	3-5 lobed lobulate		2.7 x 2.3 cm .1-.5 cm spines	.50 x .22 x .12
275571	C. africanus	24	3-5 lobed lobulate		4.7 x 2.7 cm .1-.8 cm spines 8/cm^2	.46 x .21 x .10
203977	C. myriocarpus	24	3-5 lobed lobulate		2.1 x 1.9 cm .1-.2 cm spines	.52 x .21 x .13
282449	C. myriocarpus	24	3-5 lobed lobulate		2.7 x 2.4 cm, .3-.5 cm spines p=4.0 cm*	.52 x .23 x .12
299568	C. myriocarpus	24	3-5 lobed lobulate	.7 x .5 cm .2 cm spines p=4.2 cm	2.8 x 2.4 cm .2-.6 cm spines	.53 x .23 x .12
374153	C. myriocarpus	24	3-5 lobed lobulate	.6 x .4 cm .2-.3 cm spines p=1.9 cm	1.6 x 1.4 cm .1-.2 cm spines	.54 x .23 x .13
282447	C. leptodermis	24	3-5 lobed lobulate			.50 x .22 x .12

*p=peduncle length.

C. myriocarpus accessions can be divided into two morphologically different groups according to their fruit characteristics. PI 299568 and 282449 have longitudinally, beige-black, striped fruits with 0.2-0.6 cm spines (Figure 21) while PI 203977 and 374153 develop light brown-dark brown striped fruits with short (0.1-0.2 cm) spines. The fruits of C. africanus also occur in two not very sharply defined forms: a large oblong shape (PI 275571) and a smaller, more ellipsoid one (PI 203975). The shapes are well correlated with taste, in that the larger fruits are a relished refreshment and source of water among African natives, whereas the smaller ones are bitter and poisonous (Meeuse, 1962).

Hybridization studies among and between C. africanus, C. leptodermis, and C. myriocarpus accessions indicated a high degree of intra- and inter-specific cross-compatibility (Table 16), and resulted in the development of vigorous and fertile F_1 hybrids with 100% 3-porate and 97% normal pollen (Table 17). Meiotic studies of pollen mother cells of (374153 x 282447) F_1 plants pointed to a high degree of chromosome homology between the speciew with the formation of 12 II in 10 PMC's.

The species and subspecies can also be separated from each other by their peroxidase banding patterns, which show isozymes at the Px_1, Px_2 and Px_3 locus (Figure 22). Peroxidases at the Px_2 locus in C. leptodermis have the same migrational and genetic behavior as those of C. melo. The Px_{2A} allele is represented by a cluster of a,

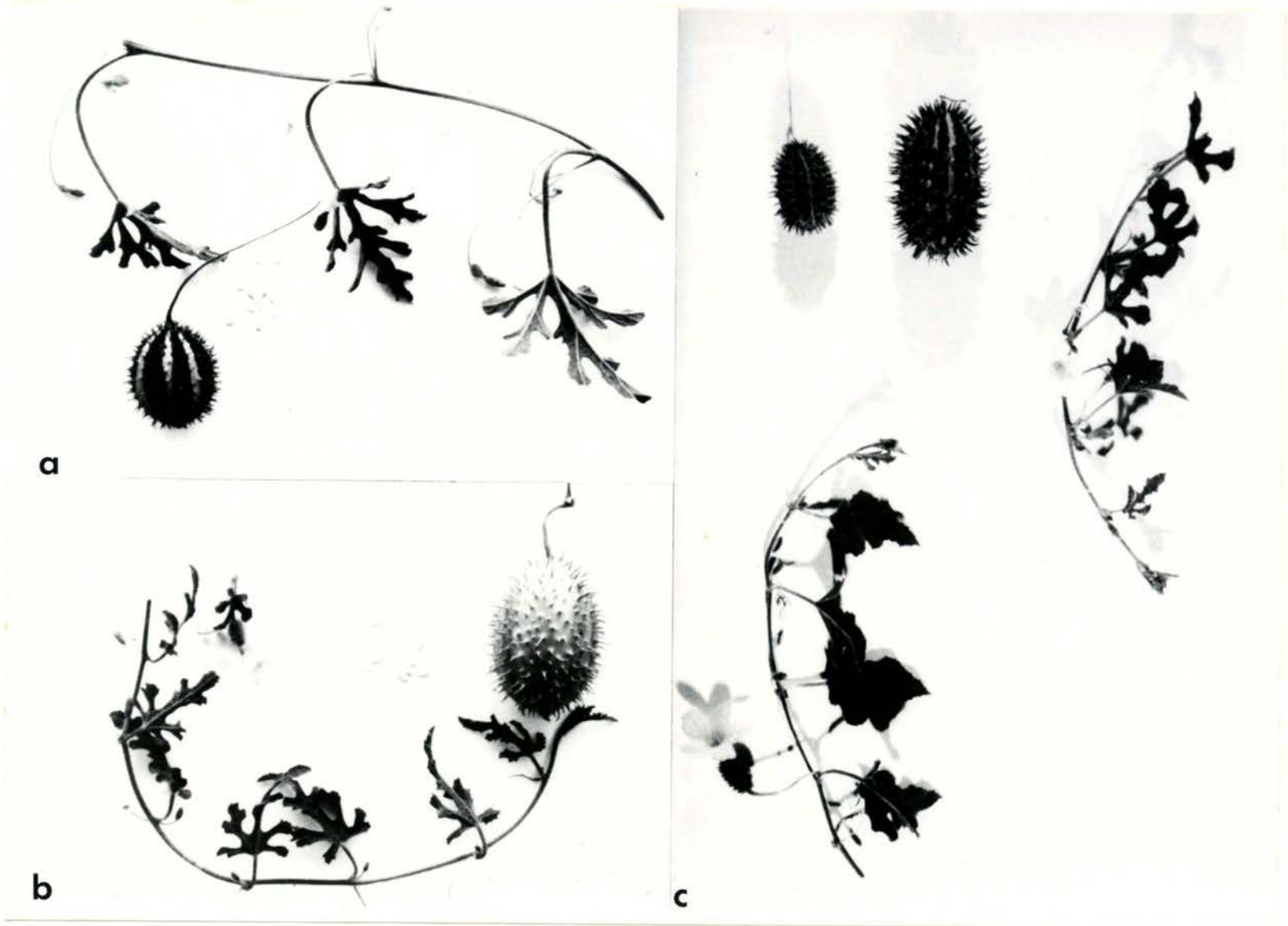

Figure 21. (a) C. myriocarpus (PI 203977), (b) C. zeyheri (PI 282450), and (c) C. africanus (PI 275571).

Table 16. Hybridization studies of Cucumis africanus, C. leptodermis and C. myriocarpus Plant Introductions.

Female Parent	Male Parent*				
	203975 275571	299568 282449	203977 374153	374151	282447
203975 275571		F	F	F	+
299568 282449	F		F		F
203977 374153	+	F		F	+
374151					
282447					

*F indicates the formation of fertile F_1 plants.
+ indicates the formation of fruits with seeds.

Table 17. Pollen morphology and fertility of C. africanus, C. leptodermis and C. myriocarpus accessions and F_1 plants.

Species or F_1	Percent Pollen		Percent Pollen	
	3-porate	4-porate	normal	aborted
C. leptodermis	97	3		
C. myriocarpus				
PI 299568	100	0		
PI 374153	99	1		
C. myriocarpus (PI 299568) x C. africanus (PI 275571)	100	0	97	3
C. myriocarpus (PI 299568x 374153) x C. leptodermis	100	0	97	3

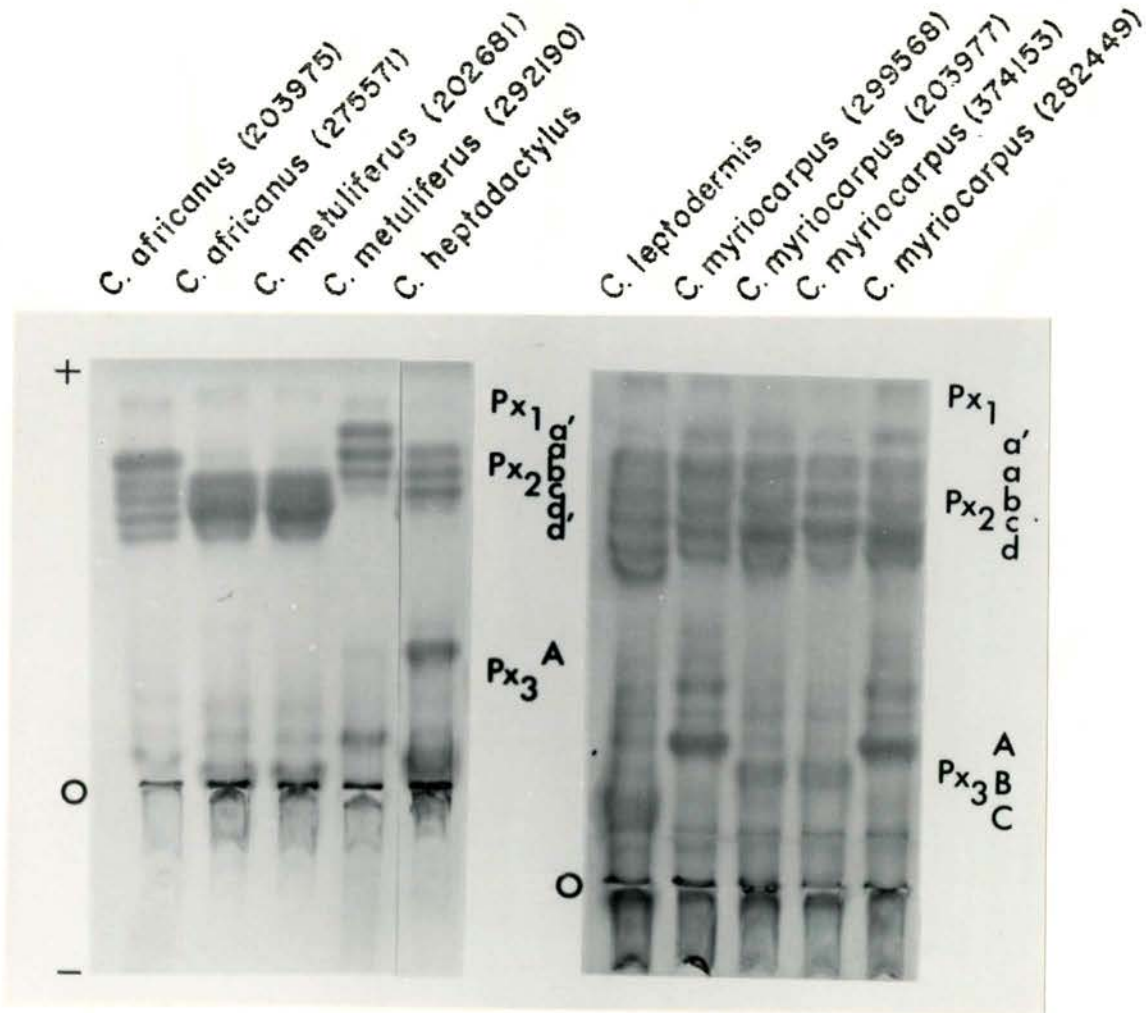

Figure 22. Peroxidase banding pattern of C. africanus, C. heptadactylus, C. leptodermis, C. metuliferus, and C. myriocarpus.

b, and c bands, and Px_{2B} by b, c and d peroxidases. Evidence for the codominant inheritance of these alleles is presented in Table 18.

Segregation at the Px_2 locus was not observed in C. myriocarpus or C. africanus accessions (Table 36). C. myriocarpus PI's showed all four (a, b, c, d) Px_2 bands although S_1 plants of PI 299568 (seven individuals) and PI 203977 (six individual plants) had only the more anodically migrating Px_{2A} cluster. C. africanus PI 374151 also had four peroxidase bands at the Px_2 locus, while PI 203975 and 275571 displayed a pattern of five (a, b, c, d, d') peroxidase bands which were in homozygous condition since five PI 275571 S_1 plants were found to have that same Px_2 cluster.

The two morphologically different C. myriocarpus forms exhibit polymorphism at the Px_3 locus. While PI 299568 and 282449 have Px_{3A}, PI 203977 and 374153 show Px_{3B}. These isozymic variants are inherited as codominant alleles at one, the Px_3, locus (Table 19).

If o-dianisidine is used as the peroxidase substrate instead of o-tolidine, PI 282449 and 299568 display Px_{3A}, but PI 203977 and 374153 do not only have Px_{3B} but also the Px_{3A} peroxidase. It is apparent that the Px_{3A} peroxidase of PI 203977 and 374153 has different H-donor specificities since it can utilize o-dianisidine but cannot use o-tolidine.

C. africanus lacks a clearcut Px_3 band definition although F_1 plants of C. myriocarpus x C. africanus (PI 275571) or its reciprocal

Table 18. Segregation of F_2 and BC generations for alleles at the Px_2 peroxidase locus in C. leptodermis.

Generations	Peroxidase Genotypes			N	X^2
	$Px_{2A}Px_{2A}$	$Px_{2A}Px_{2B}$	$Px_{2B}Px_{2B}$		
F_2		4	2	6	2.0
F_2	3	7	3	13	0.1
$Px_{2A}Px_{2B} \times Px_{2A}Px_{2A}$	2	4		6	0.7
$Px_{2A}Px_{2B} \times Px_{2A}Px_{2A}$	3	1		4	1.0

Table 19. Segregation of F_2 and BC generations for peroxidase alleles at the Px_3 locus in C. myriocarpus.

Generations	Peroxidase Genotypes			N	X^2
	$Px_{3A}Px_{3A}$	$Px_{3A}Px_{3B}$	$Px_{3B}Px_{3B}$		
F_2	3	7	2	12	0.5
$Px_{3A}Px_{3B} \times Px_{3A}Px_{3A}$	1	3		4	1.0

show not only Px_{3A} or Px_{3B} but also the Px_{3C} peroxidase. Inheritance studies of the Px_{3C} peroxidase have been carried out with F_2 seedlings but failed to show Px_3 peroxidase band definition and led to the conclusion that the Px_3 alleles are developmentally determined.

The close relationship between the annual South African species is also indicated by their peroxidase (Figure 23) and esterase (Figure 34) isozyme patterns developed upon isoelectric focusing of enzymes extracted from these species. Both supported the classification of PI 374151, whose plants failed to develop pistillate flowers, in C. africanus since the esterase and peroxidase isoelectric focusing pattern of PI 374151 and 275571 were identical. The relationship of these species with other members of the genus will be discussed in the "Relationship Between the Wild and Cultivated Cucumis Species" section of this chapter.

Cucumis heptadactylus

This dioecious perennial and tetraploid species is distinctly different from all other Cucumis species in that it develops deeply dissected leaves with very long and narrow linear lobes (Figure 25a). Deakin et al. (1971) reported that C. heptadactylus produced fruits, lacking viable seeds, in crosses with C. myriocarpus but that it failed to set fruit with eight other Cucumis species. Since the C. heptadactylus plants failed to produce pistillate flowers, they could only be used as pollen parent and were found to be cross-incompatible

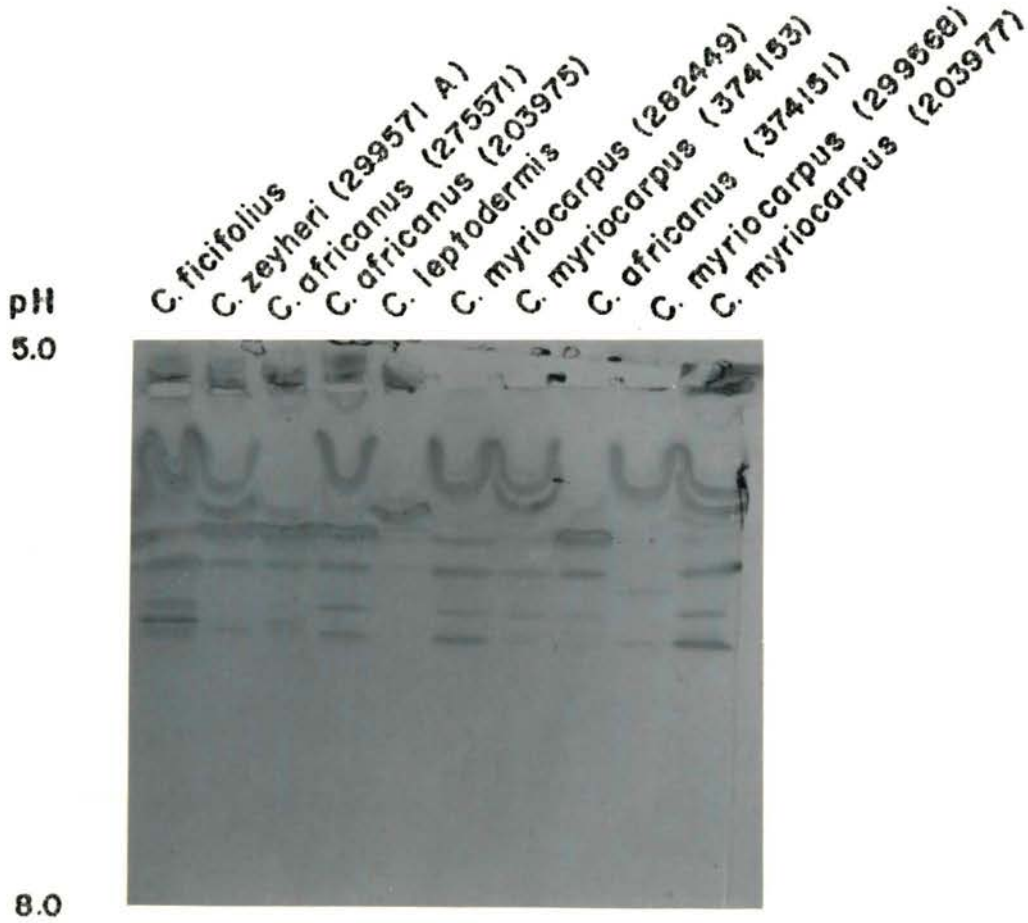

Figure 23. Peroxidase isoelectric focusing pattern of C. africanus, C. ficifolius, C. leptodermis, C. myriocarpus and C. zeyheri (299571 A).

with C. africanus, C. leptodermis and C. myriocarpus. It should be mentioned, however, that these annual South African species exhibit crossing barriers with all other Cucumis species (Table 29).

Meiotic chromosome studies indicate the autotetraploid nature of C. heptadactylus since a maximum of ten quadrivalent chromosome configurations were observed in pollen mother cells of this species (Table 20, Figure 24). These results confirm Shimotsuma's (1965) chromosome studies, in which VI, IV, III, II and I were observed. Tetraploidy was accompanied by an increase in the number of four or more porate pollen grains, since 62% of the pollen were found to be 3-porate and 38% 4-porate, but not by a large increase in the number of aborted pollen (only 8%).

The peroxidase banding pattern of this species, given in Figure 22, shows isozymes at the Px_1 locus, a cluster of very anodically migrating Px_2 bands and the Px_{3B} peroxidase. Individual C. heptadactylus exhibit a cluster of three a'ab or abc, or four a'abc bands at the Px_2 locus indicative of an allelic peroxidase isozyme behavior like that in C. melo and C. leptodermis (Table 36).

Similarities between the autotetraploid and C. africanus, C. leptodermis and C. myriocarpus were apparent in their GOT isozyme, flavonoid and peroxidase isoelectric focusing patterns. Although C. africanus is considered as an annual species (Meeuse, 1962), it produces tuberous roots and has been grown in the greenhouse for a period of two years. The results indicate that these

Table 20. Meiotic chromosome configurations at diakinesis and metaphase I in Cucumis heptadactylus (2n=48).

Chromosome Configurations IV	II	Number of Pollen Mother Cells
	24	9
1	22	11
2	20	12
3	18	10
4	16	3
5	14	3
6	12	1
7	10	1
10	4	1
2.24 IV	19.5 II	$\bar{x}$

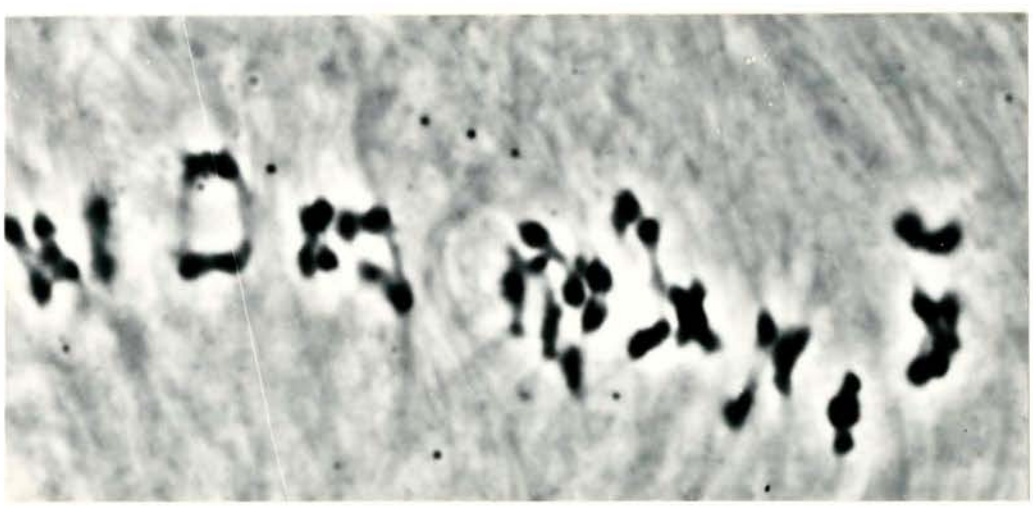

Figure 24. Meiotic chromosome configurations at metaphase I in Cucumis heptadactylus showing 10 IV + 4 II (4000x).

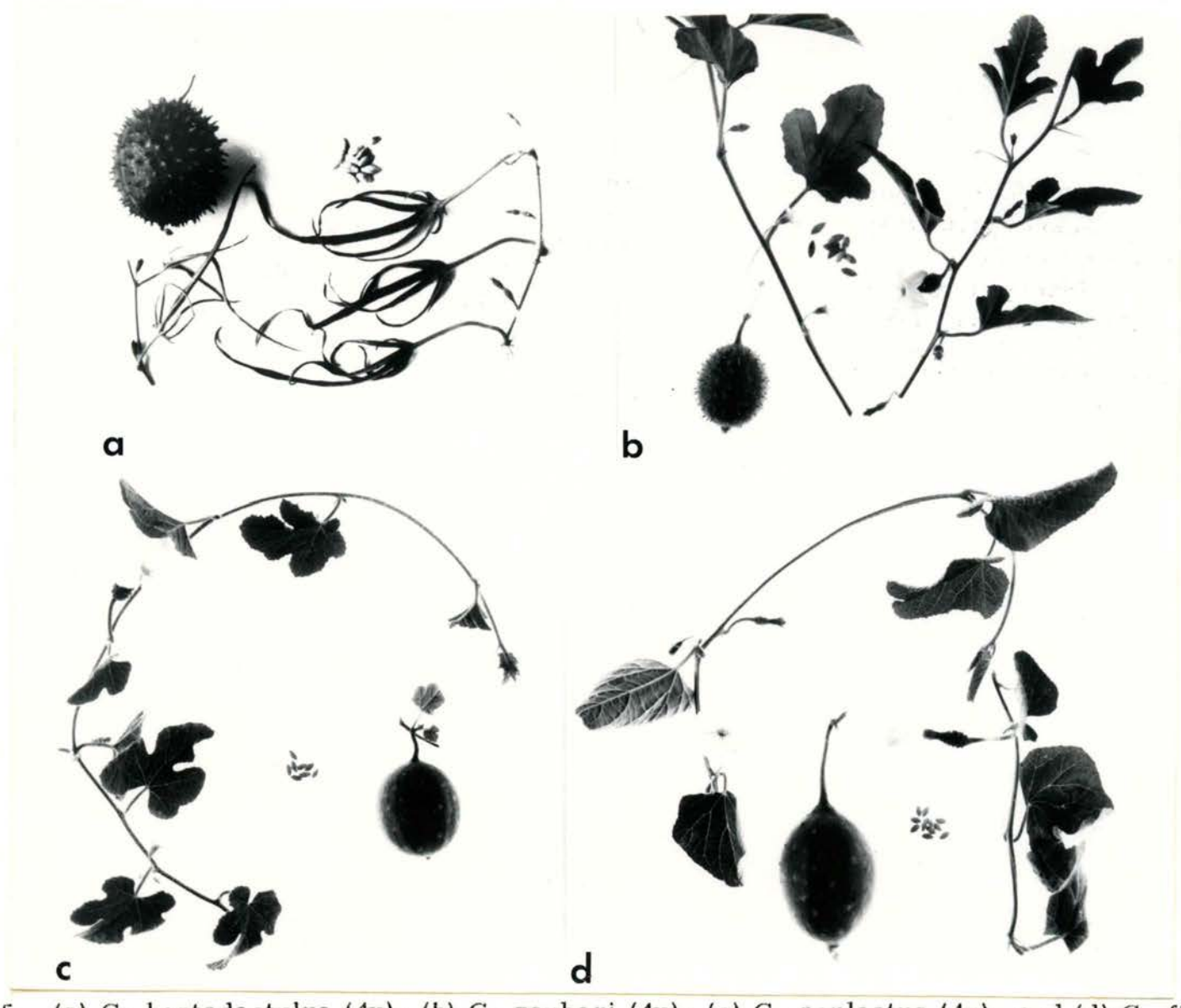

Figure 25. (a) C. heptadactylus (4x), (b) C. zeyheri (4x), (c) C. aculeatus (4x), and (d) C. figarei (6x).

three annual diploids and perennial autotetraploid are very closely related.

Cucumis zeyheri

Meeuse (1962) describes C. zeyheri as a perennial, monoecious spiny-fruited herb distributed in South Africa, South Rhodesia, and East Africa but not in South West Africa and Angola. C. prophetarum (Jeffrey, 1967) is morphologically similar to C. zeyheri (Meeuse, 1962) but indigenous to East Africa. Accessions labeled as C. africanus or C. zeyheri (PI 203974, 274036, 299569, 282450, 315212, 364473, 299570, 273192, 299571 and 299572) were collected in Southern Africa (Figure 20) and have many morphological characteristics in common with C. zeyheri (Meeuse, 1962). Their leaf, ovary, fruit and seed morphology and chromosome number are reported in Table 21 and 25. All develop light green/dark green, longitudinally striped fruits which yellow upon maturation. PI 203974, 274036, 282450, 299569, 315212 and 364473 are diploid and PI 273192, 299570, and 299572 are tetraploid (Dane and Tsuchiya, 1976).

The diploid accessions are not only morphologically similar but hybridize quite easily following hand pollination, produce vigorous F_1's (Table 22), and have an identical peroxidase isozyme pattern characterized by isozymes at the Px_1, Px_2 (a', a, b, c, d) and Px_3(C) loci (Figure 26, Table 36). Pollen mother cells of PI 364473 show normal bivalent chromosome configurations (26 PMC's with 12 II, 1

Table 21. Morphological characteristics and chromosome number of Cucumis zeyheri Plant Introductions.

Number	2n	Leaf Morphology	Ovary Morphology	Fruit Morphology	Seed Size in cm
203974	24	deeply 3-5 lobed lobulate		5.3x3.5 cm, 0.1-0.6 cm spines, 7.7/cm^2, p=2.5 cm*	.77x.34x.17
274036	24	deeply 3-5 lobed lobulate		3.3x2.3 cm, 0.1-0.6 cm spines, 10.3/cm^2, p=1.2 cm	.67x.30x.17
299569	24	deeply 3-5 lobed lobulate		4.4x3.4 cm, 0.2-0.4 cm spines, 15/cm^2, p=1.4 cm	.74x.35x.16
282450	24	deeply 3-5 lobed lobulate		4.4x3.3 cm, 0.2-0.8 cm spines, 7/cm^2, p=2.0 cm	.74x.34x.17
315212	24	deeply 3-5 lobed lobulate	1.2x0.6 cm 0.2 cm spines	4.2x3.0 cm, 0.2-0.9 cm spines, 9.3/cm^2, p=2.3 cm	.72x.34x.16
364473	24	deeply 3-5 lobed lobulate	1.2x0.6 cm, 0.2-0.3 cm spines	3.6x2.6 cm, 0.2-0.8 cm spines	.64x.25x.14
273192	48	slightly 3-5 lobed	0.9x0.4 cm, 0.1 cm spines, p=1.6 cm	3.0x2.7 cm, 0.1-0.2 cm spines, 20/cm^2, p=1.2 cm	.78x.32x.15
299570	48	slightly 3-5 lobed	1.0x0.5 cm, 0.1 cm spines, p=1.8 cm	2.7x2.3 cm, 0.1-0.2 cm spines, 12/cm^2, p=1.5 cm	.73x.32x.15
299572	48	slightly 3-5 lobed			.69x.20x.15

*p = peduncle length.

Table 22. Hybridization studies of Cucumis zeyheri Plant Introductions.

Female Parent	Male Parent*								
	203974	274036	282450	299569	315212	364473	273192	299570	299572
203974		F							
274036	+				F				
282450		F			+	+		+	
299569			F			F			
315212			F			F			
364473		+			+			S	
273192	+		+					+	
299570	F		+	+			+		
299572								+	

*F indicates the formation of vigorous F_1 plants.
+ indicates the production of fruit and seed upon cross-pollination.
S indicates the production of fruit lacking viable seed upon cross-pollination.

PMC with 11 II and 2 I) and develop only 3-porate pollen grains. These results indicate that the diploid C. zeyheri accessions are conspecific.

The tetraploid accessions (PI 273192, 299570 and 299572) are morphologically similar, cross-compatible with each other (Table 22) and characterized by peroxidases at the Px_1, Px_2 (a, b, c, d, d') and Px_3 (B and C) loci (Figure 26, Table 36). Meiotic chromosome studies of PI 299570 showed 24 bivalents in ten pollen mother cells (Figure 27) indicating that the chromosome complement of the accession has two different genomes. More support for this conclusion comes from pollen morphological studies (Table 23). Almost no 4-porate or aborted pollen were observed in staminate flowers from PI 273192 and 299570.

Table 23. Pollen morphology and fertility of tetraploid Cucumis zeyheri accessions.

C. zeyheri PI	Percent Pollen		Percent Pollen	
	3-porate	4-porate	normal	aborted
273192	99	1	99	1
299570	99	1	99	1

The diploid and tetraploid C. zeyheri accessions are cross-compatible (Table 22) and produce vigorous triploid F_1 plants which show 8-12 bivalent and 0-3 trivalent chromosome configurations in their pollen mother cells (Table 24, Figure 28). A high percentage

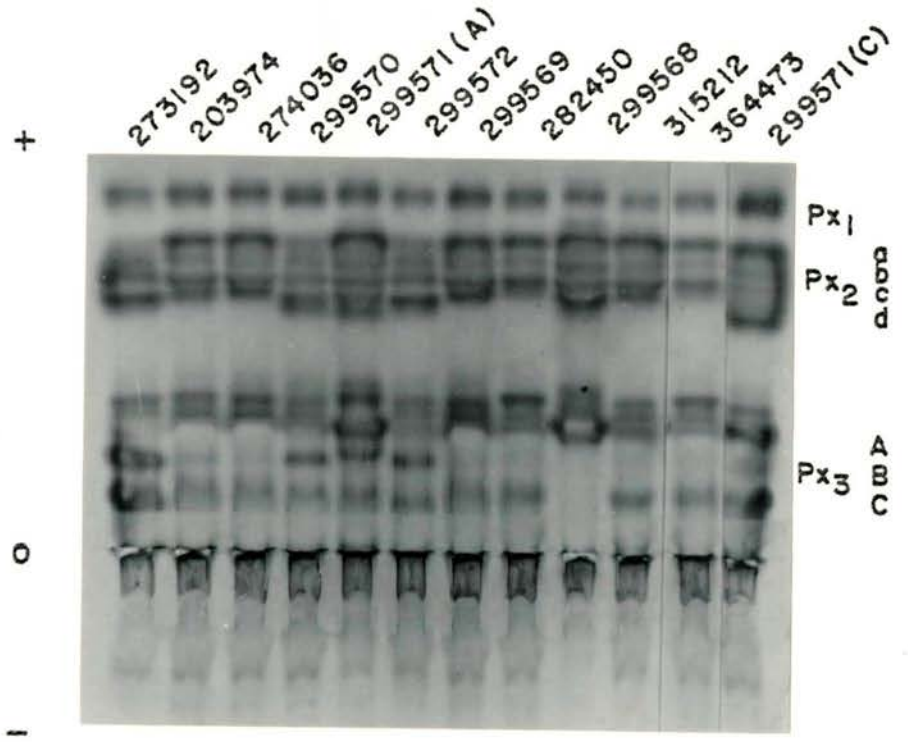

Figure 26. Peroxidase banding pattern of the diploid and tetraploid Cucumis zeyheri accessions.

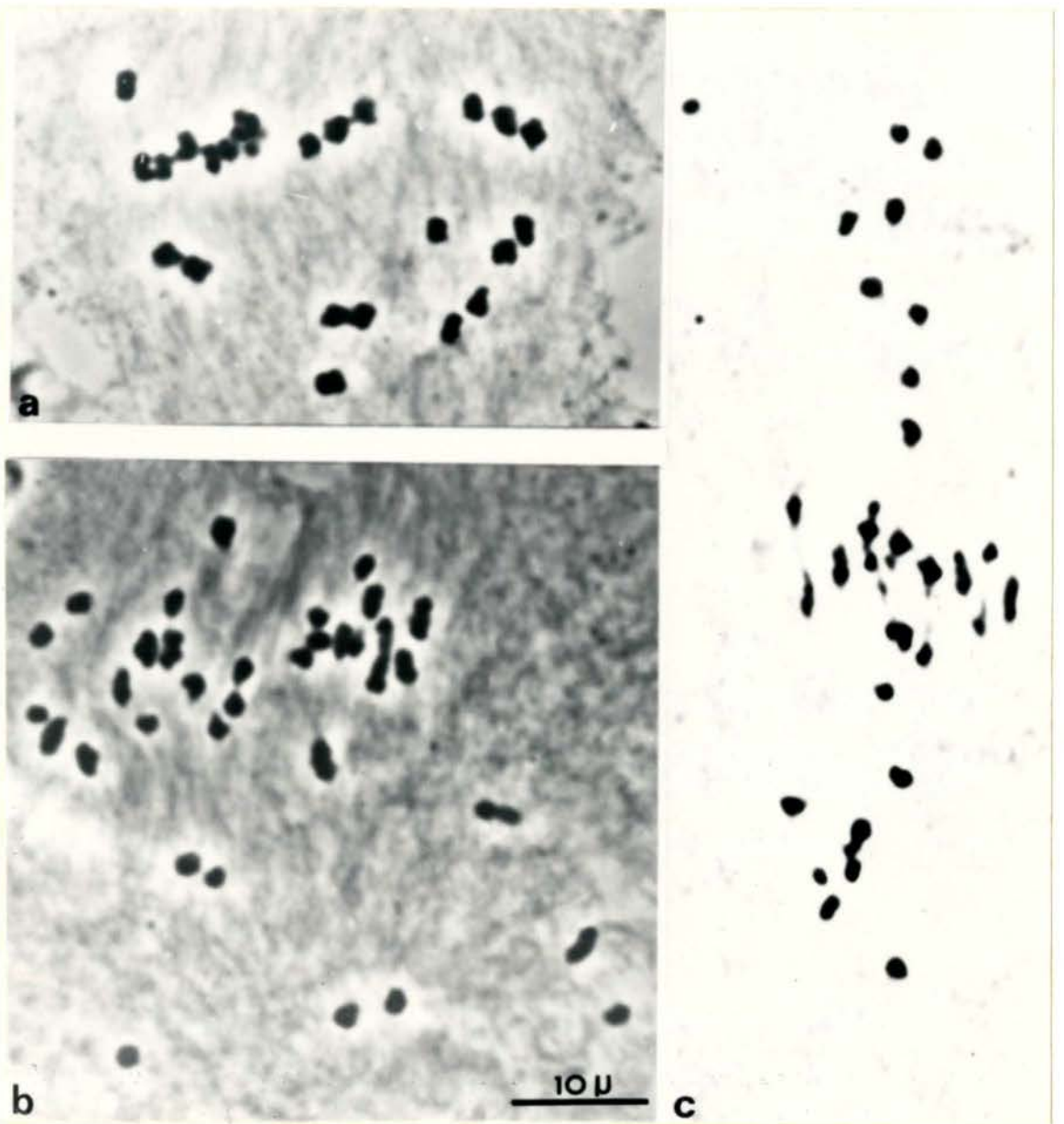

Figure 27. Meiotic chromosome configurations at metaphase I of
a. the tetraploid C. zeyheri PI 299570 with 24 II
b. the tetraploid C. zeyheri PI 299571 C with 15 II + 18 I
c. the triploid C. zeyheri PI 299571 A with 10 II + 16 I

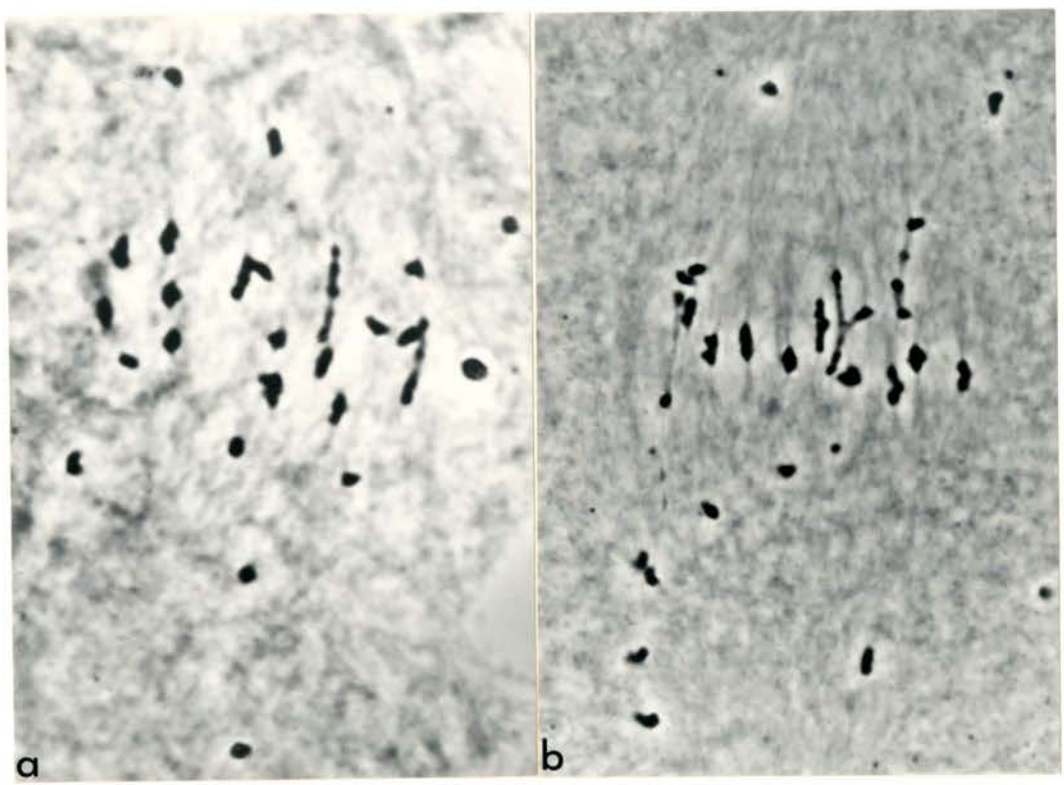

Figure 28. Meiotic chromosome configurations at metaphase I in triploid Cucumis zeyheri (PI 299570) x C. zeyheri (PI 203974) F_1 plants (1500x).
a. 2 III + 9 II + 12 I
b. 2 III + 10 II + 10 I

Table 24. Meiotic chromosome configurations at metaphase I and diakinesis in triploid F_1 plants of Cucumis zeyheri PI 299570 x PI 203974.

Chromosome Configurations			Number of
III	II	I	Pollen Mother Cells
	12	12	10
1	11	11	6
3	9	9	1
	11	14	6
1	10	13	1
2	9	12	2
	10	16	2
2	8	14	1
1	8	15	1
0.6 III	10.8 II	12.6 I	$\bar{x}$

of the pollen produced by the triploid (299570 x 203974) contained four or more pores (35%) or were aborted (59% of the pollen), the result of abnormal chromosome segregation and the formation of unreduced gametes.

Isoelectric focusing of the enzymes extracted from the diploid and tetraploid accessions and staining for peroxidases or esterases produced almost identical isozyme patterns (Figure 29 and 34). The high degree of similarity between the diploid and tetraploid accessions in their isozyme patterns and morphology, and the high frequency of bivalent chromosome configurations in their triploid F_1 hybrids led to

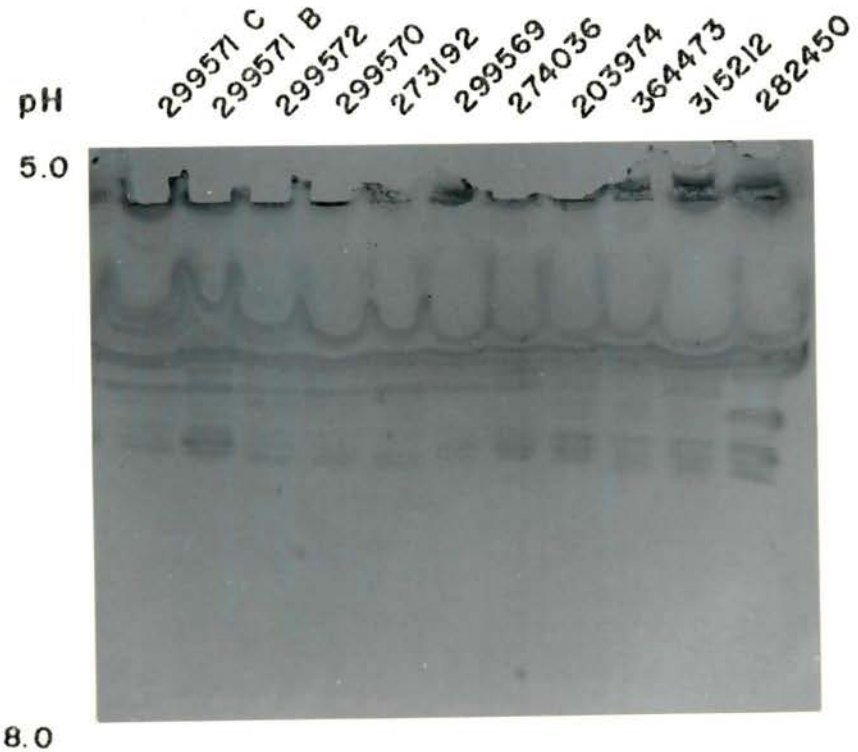

Figure 29. Peroxidase isoelectric focusing pattern of C. zeyheri accessions.

the conclusion that the diploid C. zeyheri served as one of the ancestors for the allotetraploid C. zeyheri.

One accession, PI 299571, was found to contain a mixture of morphologically, cytologically and biochemically different plants (Table 25). Form A, a perennial triploid, is morphologically related to C. leptodermis, while form B, a perennial tetraploid, is similar to the tetraploid C. zeyheri and form C, also a perennial tetraploid, is closer related morphologically to the diploid C. zeyheri.

Pollen mother cells of PI 299571 form B (four cells) showed 24 bivalents, produced 98% 3-porate, 2% 4-porate, 98% normal and 2% aborted pollen like pollen mother cells of 273192 and 299570 (Table 23). The plants were self-compatible and cross-compatible with C. myriocarpus, C. africanus, the diploid and tetraploid C. zeyheri, C. ficifolius and PI 299571 form C. Their peroxidase banding pattern is similar to that of the triploid PI 299571 form A. It is characterized by bands at the Px_1, 5-6 bands at the Px_2 and 1-3 bands at the Px_3 locus (Figure 26). Since the morphological studies show similarity between PI 299571 form B and the other tetraploid C. zeyheri (PI 273192, 299570 and 299572) accessions, the occurrence of Px_{3A} in the tissues of PI 299571 form B can be explained by a mutation in one of the peroxidase isozymes or the presence of Px_{3A} (instead of Px_{3B}) in one of its ancestors.

The meiotic chromosome behavior of form A, outlined in Table 26, Figure 27, indicates that the plants are the offspring of a natural

Table 25. Morphological characteristics and chromosome number of Cucumis zeyheri PI 299571 form A, B and C.

Form	2n	Leaf Morphology	Ovary Morphology	Fruit Morphology	Seed Size (cm)
A	36	3-5 lobed, lobulate	.7 x .4 cm with .1 cm spines	2.2 x 1.6 cm with 0.1 cm spines	.64 x .31 x .15
B	48	3-5 lobed		2.6 x 2.2 cm with .1-.2 cm spines	.68 x .31 x .15
C	48	deeply 3-5 lobed lobulate		3.0 x 2.0 cm, with .2-.4 cm spines	

Table 26. Meiotic chromosome behavior of C. zeyheri PI 299571 form A (2n=36).

Chromosome Configurations II	Chromosome Configurations I	Number of Pollen Mother Cells
12	12	4
11	14	1
10	16	1
9	18	2
8	20	1
7	22	1
10.2 II	13.8 I	$\bar{x}$

cross between an allotetraploid and diploid, which have one genome in common. The plants produced fruits with 1-2 seeds when crossed with C. myriocarpus and C. leptodermis pollen, fruits without seeds when crossed with Cucumis africanus, C. anguria, C. dipsaceus, and the diploid C. zeyheri but were self-incompatible, and cross-incompatible with other Cucumis species. The triploidy was associated with a high percentage of four or more porate pollen (20% 3-porate; 71% four or more porate) and aborted pollen (16% abnormal; 84% normal). The presence of Px_{3A} in its peroxidase banding pattern points to the diploid C. myriocarpus or C. anguria var. anguria or the tetraploid C. zeyheri PI 299571 form B. Only studies of F_1 plants between C. zeyheri PI 299571 form B or other tetraploid C. zeyheri accessions and C. myriocarpus, C. leptodermis or C. anguria var. longipes can provide conclusive evidence for the most

likely suggestion (based on morphology and peroxidase isozyme patterns) that C. leptodermis and PI 299571 form B were the ancestors of the triploid PI 299571 form A.

Since PI 299571 form C was self-incompatible but cross-compativle with C. africanus, C. anguria var. longipes, C. myriocarpus and C. zeyheri (2x), but produced sterile fruits with C. zeyheri (4x) pollen, its meiotic chromosome behavior was studied. While an average of two trivalents and 14 bivalents were observed in pollen mother cells of these plants, a maximum of 8 III was counted in one cell and a maximum of 20 II in another (Table 27, Figure 27). A genome formula of AAA'A' or AAAA' can explain this unusual meiotic behavior. It was accompanied by the production of multiporate pollen grains (83% $\geqslant$ 4-porate and 17% 3-porate) and pollen abortion (18% aborted pollen, 82% normal). The peroxidase banding pattern of PI 299571 form C plants is shown in Figure 26. A cluster of six (a'abcdd') Px_2 bands and three Px_3 (A, B, and C in a low frequency) peroxidases were observed. The peroxidase banding pattern developed upon isoelectric focusing of its plant juice looks like the C. zeyheri (2x or 4x) patterns with some additional low pI bands observed in C. africanus, C. anguria, C. ficifolius or C. myriocarpus. The peroxidase isoelectric focusing pattern of F_1 plants from C. anguria var. longipes x C. africanus is identical to that of PI 299571 form C x C. anguria var. longipes (Figure 29). Several hypotheses can be proposed to explain the evolution of this unusual tetraploid. It may

Table 27. Meiotic configurations at diakinesis and metaphase I in Cucumis zeyheri PI 299571 form C (2n=48).

Chromosome Configurations			Number of
III	II	I	Pollen Mother Cells
2	20	2	1
2	19	4	1
5	15	3	1
6	14	2	1
8	11	2	1
	17	14	4
3	14	11	1
2	14	14	6
2	13	16	1
	15	18	1
3	11	17	1
2	12	18	1
1	13	19	1
2	11	20	1
1	11	14	3
2.1 III	14.12 II	12.6 I	$\bar{x}$

have resulted from chromosome doubling of the diploid: C. zeyheri (with genome formula AA) x C. anguria var. longipes (genome formula A'A'), and have a genome formula of AAA'A'. However, quadrivalents should then be produced at metaphase I, and C. anguria var. longipes should have contributed the Px_{3A} and Px_{3B} peroxidases. A genome formula of AAAA' seems to be more likely as far as the meiotic chromosome behavior of the plants is concerned and points

to an autotetraploid (AAAA, C. heptadactylus?) in their ancestry. More detailed chromosome, isozyme and morphological analyses of several PI 299571 plants and intra- and inter-specific F_1's are needed to solve the ancestry of PI 299571 form C plants.

Cucumis metuliferus

C. metuliferus, a native of Southern Africa, is known as the "jelly melon" for its jelly-like sacs which individually envelop the hairy seeds or as the "horned cucumber" for its ringmottled orange fruit with prominent fleshy spines (Figure 30). The species is diploid (2n=24 chromosomes), monoecious, annual, and cross-incompatible with 14 other Cucumis species (Deakin et al., 1971). Crosses between this species and C. melo were attempted since C. metuliferus is highly resistant to the watermelon mosaic virus, squash mosaic virus, and root knot nematode, but were unsuccessful (Provvidenti and Robinson, 1974). It was used unsuccessfully as a pollen parent in crosses with C. aculeatus, C. africanus, C. ficifolius, C. figarei and the diploid C. zeyheri.

The distinctness of this Cucumis species was also evident in its peroxidase banding pattern, in that it contains a displayed Px_1 band, Px_2a', and a cluster of bands with the same electrophoretic mobility as the Px_2 cluster of C. sativus (Figure 22). The two Plant Introductions (202681 and 292190) are morphologically similar and show the same peroxidases upon electrophoresis. The C. metuliferus

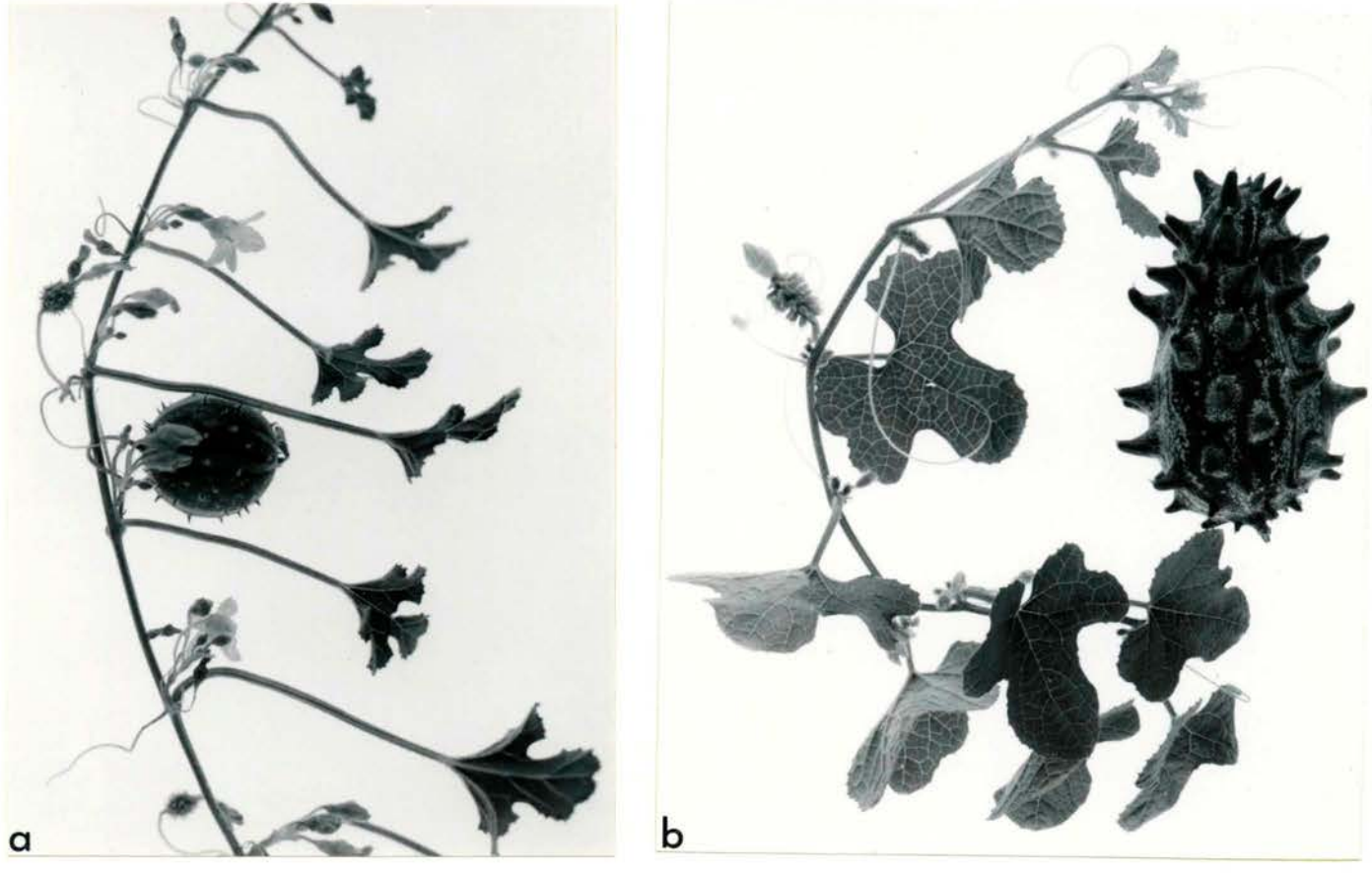

Figure 30. (a) C. leptodermis, and (b) C. metuliferus.

peroxidase banding pattern is in homozygous condition since six S_1 plants showed a pattern of Px_1', Px_2 a' and a cluster of Px_2 bcd. The peroxidase isozyme pattern obtained after isoelectric focusing of C. metuliferus plant extract also showed one unusual isozyme (with an isoelectric point of approximately 6.4; Figure 33) which is also found in C. sativus. Examination of the GOT patterns of the Cucumis species showed that C. metuliferus has three of its four GOT isozymes in common with C. melo, C. asper, and C. dinteri, and only two with all other species. Flavonoid studies by Brown et al. (1969) indicated that C. metuliferus shared three of its four flavonoids with C. sativus and C. africanus and two or less with the other Cucumis species. An overall similarity index calculated from all these isozyme and flavonoid data showed the highest similarity between C. metuliferus and C. sativus (0.46, Table 37), but this index weights all isozymic bands and flavonoids equally and probably overestimates the similarity between the two widely separated species.

Cucumis angolensis, C. dinteri and C. sagittatus

These perennial, monoecious and diploid (2n=24, Dane and Tsuchiya, 1976) species are closely related morphologically and biochemically. Differences do exist in their fruit morphology and cucurbitacin D content. While the fruits of C. angolensis are ellipsoid, longitudinally variegated, pale yellow and have only traces of cucurbitacin D, fruits of C. dinteri are subglobose, deep yellow to

orange and very bitter (high content of cucurbitacin D) (Meeuse, 1962). Hybridization studies between C. dinteri and C. sagittatus (C. angolensis could never be studied beyond the seedling stage) indicated interspecific cross-compatibility. The species were cross-incompatible with C. anguria var. anguria, C. ficifolius, C. africanus, C. heptadactylus, C. myriocarpus and failed to set fruits as pollen parent in crosses with C. aculeatus, C. africanus, C. anguria, C. ficifolius and the diploid C. zeyheri. These results confirm Deakin et al. (1971) hybridization studies, which also failed to break the incompatibility barrier between these and the other Cucumis species.

Their peroxidase isozyme patterns were very similar, although differences existed in the frequency of the Px_{3A} and Px_{2} d bands (Table 36). This similarity in pattern was also demonstrated in the peroxidase banding patterns obtained after isoelectric focusing of the enzymes extracted from the C. dinteri and C. sagittatus plant tissues. The hybridization and zymogram data support Deakin et al. (1971)'s conclusions that the species are conspecific.

The relationship of C. dinteri to other Cucumis species was studied by comparison of their zymogram and flavonoid data. C. dinteri had all of its GOT isozymes in common with C. melo and C. metuliferus, but four of its five flavonoids with C. anguria. An overall similarity index calculated from all isozyme and flavonoid data

indicated that the species was closest related to C. anguria (similarity index = .59, Table 37).

Cucumis asper

Meeuse (1962) describes C. asper as a perennial dioecious species with dentate 5-lobed leaves, unique asperulous fruits and hairy seeds growing in rock crevices of South West Africa. The species was found to be diploid (2n=24 chromosomes, Dane and Tsuchiya, 1976). It failed to produce pistillate flowers and was cross-incompatible with C. aculeatus and C. anguria.

Its peroxidase isozyme pattern lacked the Px_1 peroxidase like C. sativus but showed a cluster of four (a'abc) bands at the Px_2 locus (Table 36). C. asper had the same three of its four GOT isozymes in common with C. sativus and C.anguria (Figure 35).

The peroxidase and esterase patterns obtained after isoelectric focusing of plant extracts from C. asper (Figure 33 and 34) show that C. asper lacks a high pI esterase common to all Cucumis species and containes a unique peroxidase band (pI around 6.4). The similarity indices calculated from all isozyme data show that C. asper is closer related to C. anguria, C. dinteri and C. sativus than to C. melo and C. metuliferus (Table 37).

Relationship Between the Wild and Cultivated Cucumis Species

The genus Cucumis can be divided into a group of relatively cross-compatible species distributed over Southern Africa, Nigeria and Ethiopia, and a group of cross-incompatible species with the cultivated Cucumis species (C. melo and C. sativus) and three species indigenous to Southern Africa. Leppik (1966) based his conclusion about the primary gene center of the genus Cucumis on phytogeographic and host-parasite relationships of the wild and cultivated Cucumis species. Many resistant C. melo Plant Introductions originated in the eastern Mediterranean area, but Indian accessions were found to contain most sources of insect and disease resistance (Appendix Table 6, Figure 10). The wild Cucumis species of both Ethiopia and South Africa are resistant to several pests and pathogens with species like C. heptadactylus and C. metuliferus of South Africa containing most of the resistant genes (Table 28). The geographical distribution of the wild Cucumis species, with species of the cross-compatible and cross-incompatible group originating in South Africa also points to South Africa as the primary gene center of the genus Cucumis.

Cross-Compatible Cucumis species

The cross-compatible group of species contains all polyploid (C. aculeatus, C. figarei, C. heptadactylus, and C. zeyheri), five annual diploid (C. africanus, C. anguria, C. dipsaceus, C. leptodermis, C. myriocarpus) and two perennial diploid (C. ficifolius and

Table 28. Wild Cucumis species with resistance to various diseases and insects.

Species	PI	Diseases or Insects*							
		Bacterial Wilt	Angular Leaf Spot	Powdery Mildew	Aphids	Mites	Root Knot Nematode	Tobacco Ring Spot Virus	Squash Mosaic Virus
C. aculeatus	193967	R	R	R	T	T			
	196844	R	R	R	T				
C. anguria	147065			R	R			R	
	196844	R		R	T	T			
	233646			R			R	R	
	282442			R			R		
C. dipsaceus	193498			R	T		T		
	236468	S	R	R	R				
C. heptadactylus	282446	R	R	R	R	R	R		
C. leptodermis	282447	R	R	R	T	T			
C. myriocarpus	282449	R	R	R	S	T	T		
C. metuliferus	202681			R	R	R	R		R
C. zeyheri	282450	R	R	R	T	T	T		

*R = resistant; T = tolerant; S = susceptible.

Data were taken from U.S.D.A., A.R.S., PI Service publications; Leppik, 1968; and Provvidenti and Robinson, 1974.

C. zeyheri) species (Table 29). Since C. heptadactylus produced fruits (without viable seeds) in crosses with C. myriocarpus (Deakin et al., 1971), it was considered a member of the cross-compatible group of Cucumis species.

The species do exhibit different degrees of cross-compatibility. The annual diploid, South African species, C. africanus, C. leptodermis and C. myriocarpus, have a strong incompatibility barrier with the polyploid species but develop fruits lacking viable seeds in crosses with the diploid C. anguria, C. dipsaceus, C. ficifolius and C. zeyheri (2x), although the reciprocal crosses were successful. C. anguria also failed to produce fruits in crosses with the polyploid species, developed sterile fruits with the diploid perennial species but vigorous F_1 plants with C. africanus and C. myriocarpus. Reciprocal crosses of the perennial diploid and allotetraploid species with C. anguria resulted in seed formation or the production of vigorous F_1 plants (Table 29). It is apparent that the annual Cucumis species developed strong cytoplasmic or embryo-endosperm incompatibility barriers with the perennial species. Whether the hybrid inviability is cytoplasmic in origin or caused by an incompatibility between the embryo and its surrounding tissue may be resolved by embryo culture (Stebbins, 1958). Stebbins (1958) also observed stronger interspecific barriers between annual than perennial species.

The low degree of cross-compatibility exhibited by the annual species was accompanied by a reduction in the number of bivalent

Table 29. Hybridization studies of the relatively cross-compatible Cucumis species.

Female Parent	Origin*	2n	Growth** Habit	Male Parent*** 1	2	3	4	5A	5B	5C	6	7A	7B	8
1 C. aculeatus	E	48	P		F	F	F	+	F	F	-	F	+	+
2 C. anguria	E, SA	24	A	-		F	S	F		F	-	S	-	
3 C. dipsaceus	E	24	A		F									
4 C. ficifolius	E	24	P	F	F	F		+	+	+		+		
5A C. africanus	SA	24	A		S	S	S		+	F	-	S	-	-
5B C. leptodermis	SA	24	A	-		-	-				-		-	
5C C. myriocarpus	SA	24	A	S	+	S	S	F	F		-	S	S	-
6 C. heptadactylus	SA	48	P											
7A C. zeyheri	SA	24	P	F	F	F	+	+	+	+			S	-
7B C. zeyheri	SA	48	P		+	F	+	+		+				
8 C. figarei	N	72	P	F		+	S	+		F		F	+	

*E = Ethiopia; SA = South Africa; N = Nigeria.

**P = perennial; A = annual.

***F = F_1 plants; S = fruit without viable seed; + = fruit with seed; - = lack of fruit formation.

chromosome configurations and pollen sterility. Although F_1 plants of C. anguria x C. dipsaceus showed 8-12 II (an average of 10.3 II, Table 14) in their pollen mother cells, the percent aborted pollen was extremely high (84%) and point to the presence of small rearrangements of chromosomal segments leading to disharmonious combinations of chromosomes in the gametes (cryptic structural hybridity; Stebbins, 1958, 1975). F_1 plants of C. anguria x C. africanus also had 8-12 II (Table 30; an average of 11.3 II per PMC), unequal chromosome distributions to the gametes resulting in 46% pollen abortion. Meiotic cells of the interspecific hybrid between C. anguria and C. myriocarpus, however, showed a considerable reduction in the number of bivalent chromosome configurations with an average of 7.1 II and a range of 3-11 II per sporocyte (Table 31), but only 15% pollen abortion. Since C. africanus and C. myriocarpus are closely related species or subspecies, chromosomal sterility can be expressed as a low amount of chromosome pairing or high degree of pollen sterility. A decrease in the number of bivalent chromosome configurations was associated with an increase in the formation of multiporate pollen grains:

F_1	avg. no. bivalents	% pollen 3-porate	% pollen 4-porate	% normal pollen
C. anguria x C. africanus	11.3	99	1	54
C. anguria x C. dipsaceus	10.3	87	13	16
C. anguria x C. myriocarpus	7.1	75	25	85

Table 30. Meiotic chromosome configurations at metaphase I in F_1 plants of C. anguria x C. africanus (PI 275571).

Chromosome Configurations II	Chromosome Configurations I	Number of Pollen Mother Cells
12		11
11	2	1
10	4	2
9	6	1
8	8	1
11.3 II	1.5 I	$\bar{x}$

Table 31. Meiotic chromosome configurations at metaphase I in F_1 plants of C. anguria (PI 282442) x C. myriocarpus (PI 203977).

Chromosome Configurations II	Chromosome Configurations I	Number of Pollen Mother Cells
11	2	1
10	4	5
9	6	4
8	8	7
7	10	6
6	12	5
5	14	3
4	16	4
3	18	2
7.1 II	9.6 I	$\bar{x}$

Perennial diploid species, C. ficifolius and C. zeyheri possess a weak incompatibility barrier with each other and the annual diploid species. F_1 plants of C. zeyheri x C. dipsaceus appeared to be heterozygous for a translocation (Table 32, Figure 31), since seven percent of their PMC's had one quadrivalent. It was associated with semi-sterility, 46% aborted and 54% normal pollen and a lack of multiporate pollen (99% 3-porate and 1% 4-porate). A similar pollen behavior was found in F_1 plants of C. anguria x C. africanus but was not accompanied by trivalent or quadrivalent chromosome configurations. Structural chromosomal differences were also apparent between the perennial diploid species, C. ficifolius and C. zeyheri, since eight to nine bivalents were counted in their interspecific F_1 pollen mother cells (Table 33).

Interspecific triploid F_1 plants between C. aculeatus and C. anguria, C. ficifolius or C. zeyheri have 0-2 III and 6-12 II (Table 14, 13 and 34) in their meiotic cells. The highest average number of bivalents but lowest average number of trivalent chromosome configurations was observed in C. aculeatus x C. ficifolius plants, while C. zeyheri appeared to have more structural chromosomal differences with the one genome of the allotetraploid C. aculeatus than C. anguria. Unequal chromosome distributions to the dividing gametes resulted in 25% pollen abortion in C. aculeatus x C. ficifolius F_1 plants, and 11% pollen abortion in C. aculeatus x C. zeyheri F_1 plants, while

Table 32. Meiotic chromosome configurations at diakinesis and metaphase I in C. dipsaceus x C. zeyheri (PI 364473) F_1 plants.

Chromosome Configurations			Number of
IV	II	I	Pollen Mother Cells
	12		26
	11 (2 pr)*		4
	8 (3 pr)		1
	11	2	23
	10	4	7
	9	6	1
	7 (2 pr)	6	1
	8	8	1
1	10		4
1	9	2	1
0.1 IV	11.2 II	1.4 I	$\bar{x}$

*pr = precocious separated bivalent.

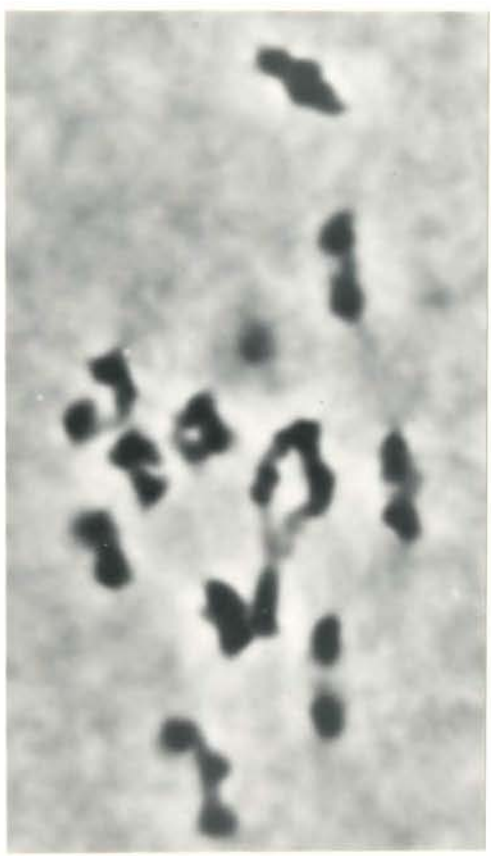

Figure 31. Meiotic chromosome configurations at metaphase I in C. dipsaceus x C. zeyheri (PI 364473) F_1 plants showing 1 IV + 10 II, 4000x.

Table 33. Meiotic chromosome configurations at metaphase I in F_1 plants of C. ficifolius (PI 280231) x C. zeyheri (PI 364473).

Chromosome Configurations II	I	Number of Pollen Mother Cells
9	6	2
8	8	4
8.3 II	7.3 I	$\bar{x}$

Table 34. Meiotic chromosome configurations at metaphase I in F_1 plants of C. aculeatus (PI 196844) x C. zeyheri (PI 364473).

Chromosome Configurations III	II	I	Number of Pollen Mother Cells
1	9	15	1
1	6	21	1
	7	22	1
	11	14	3
0.3 III	9.2 II	16.7 I	$\bar{x}$

F_1	avg. no. bivalents	avg. no. trivalents	% pollen 3-porate	% pollen normal
C. aculeatus x C. anguria	10.2	0.3	40	
C. aculeatus x C. ficifolius	11.8	0.1	96	75
C. aculeatus x C. zeyheri	9.2	0.3	79	89
C. zeyheri (4x) x C. zeyheri (2x)	10.8	0.6	64	41

the highest number of unreduced gametes or multi-porate pollen grains were observed in C. aculeatus x C. anguria F_1 plants. A similar meiotic chromosome behavior was noted in F_1 plants between the allotetraploid and diploid C. zeyheri (Table 24), which indicates that the diploid species have one genome in common with both allotetraploids.

Cucumis figarei, the allohexaploid, is cross-compatible with the diploid and tetraploid Cucumis species and forms an average of 0.3 trivalent and 12.2 bivalents in F_1 plants of C. figarei x C. aculeatus (Table 35, Figure 32). These pentaploid plants develop a high percentage (83%) of multiporate and aborted (49%) pollen. Since more than 12 bivalents were observed in PMC's of C. figarei x C. aculeatus plants, the species have two genomes in common, one of which is

Table 35. Meiotic chromosome behavior of pentaploid F_1 plants of Cucumis figarei x C. aculeatus (273649).

Chromosome Configurations III	II	I	Number of Pollen Mother Cells
1	18	21	1
	17	26	1
	12	36	3
1	11	35	2
1	10	37	1
	10	40	1
	9	42	1
0.3 III	12.2 II	34.4 I	$\bar{x}$

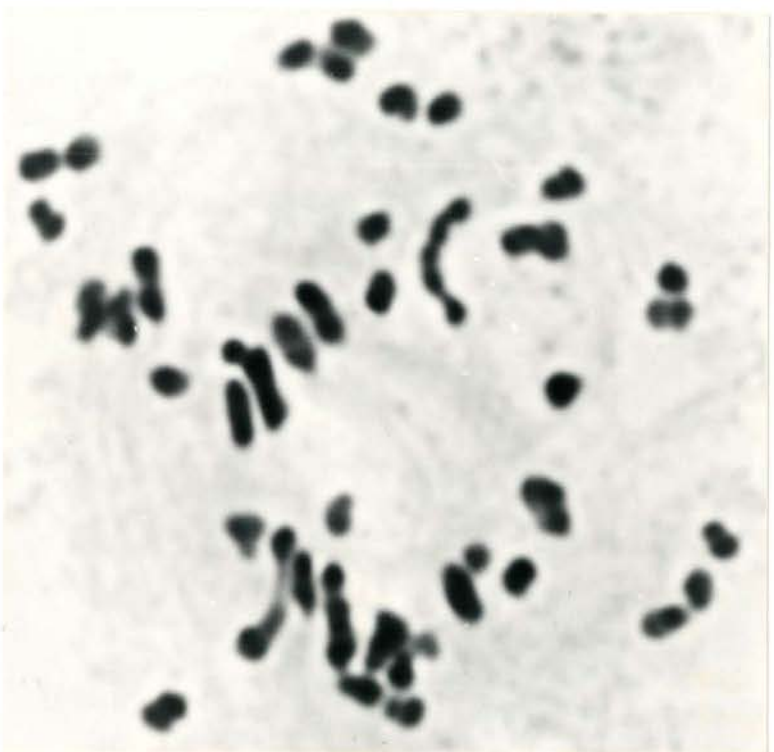

Figure 32. Meiotic chromosome configurations at metaphase I in pollen mother cells of C. figarei x C. aculeatus showing 1 III + 18 II + 21 I (3000x).

homologous to the genome of the diploid cross-compatible species. More evidence for the presence of two homologous genomes in C. figarei was indicated by the relatively high percentage of 4-porate pollen (26%), and the presence of lagging chromosome configurations in anaphase I cells. Whether C. zeyheri (4x) also has two genomes in common with C. figarei remains to be investigated. Crosses between C. figarei and C. zeyheri (4x) resulted in seed formation, but the seeds failed to germinate.

Polyploidy combined with hybridization has exerted a major influence on the evolution of plant species (Stebbins, 1971) and is not as rare in the genus Cucumis as originally thought (Kozuchov, 1930; Shimotsuma, 1965). The spontaneous formation of polysomatic cells, known to occur regularly in the muskmelon (Ervin, 1939, 1941) and found in root tip cells of the wild Cucumis species or the production of unreduced gametes, detectable as multiporate pollen in various diploid hybrids, may have caused the formation of polyploid plants. It seemed to be maintained only in perennial plants since all naturally occurring polyploid Cucumis species are perennial. Two of the polyploid species have efficient vegetative reproduction systems. In C. aculeatus rooting occurs frequently at nodes, while C. heptadactylus develops sprouts from tuberous roots. This supports Stebbins' hypothesis (1971) that successful polyploids are usually long-lived perennial species with efficient vegetative reproduction systems needed to overcome the initial stages of semi-sterility.

Allotetraploidy was not associated with the development of multi-porate pollen while the autotetraploid Cucumis species produced 38% 4-porate, and the allohexaploid 26% multiporate pollen grains. However, Funke (1956) and Maurizio (1956) observed an increase in the number of pores per pollen grain with an increase in ploidy level of both allo- and autoploids, although the increase was less pronounced in alloploids as compared to autoploids.

Chromosomal rearrangements, also found in other genera of the Cucurbitaceae (Weiling, 1959; Shimotsuma, 1963), have affected the evolution of the Cucumis species. Only one interspecific hybrid was characterized by heterozygosity for a translocation, but reduced meiotic chromosome pairing and pollen sterility in interspecific F_1 plants point to the presence of small chromosomal rearrangements or cryptic structural hybridity (Stebbins, 1958). Additional meiotic chromosome studies are needed to uncover more chromosomal rearrangements and develop an evolutionary pathway for the diploid Cucumis species. C. anguria and C. dipsaceus may have evolved from the same perennial ancestor (C. ficifolius or C. zeyheri?) and analyses of meiotic chromosome configurations of interspecific F_1 hybrids between C. zeyheri or C. ficifolius and C. anguria or C. dipsaceus may provide an answer.

Species of the cross-compatible group show a high degree of similarity in their isoperoxidase banding pattern. Most species have a similar Px_2 cluster of bands (Figures 6, 11, 13, 16, 22; Table 36)

Table 36. Frequency of peroxidase isozymes in leaf and stem tissues of the wild and cultivated Cucumis species.

Species	PI Number	Peroxidase Band Frequency at the Locus										
		Px_1	Px_2						Px_3			
			a'	a	b	c	d	d'	A	B	C	N*
C. aculeatus	193967	.8		1.0	1.0	1.0	.6	.2	.4	.6		5
	196844	.4	.1	1.0	1.0	1.0	.7	.1	.1	.8		15
	273648	.5	.1	.8	.8	.8	.5	.5	.2	1.0	.1	15
	273649	.5	.4	1.0	1.0	1.0	.6	.3	.4	.8		8
	273650	.7	.4	1.0	1.0	1.0	.6	.1	.1	.4	.1	28
C. anguria	147065	.3	.3	.3	.3	.3	.3			1.0		3
	196477	.6	.4	1.0	1.0	1.0	1.0		.1	.6		13
	233646	.7	.4	1.0	1.0	1.0	1.0			.9		27
	320052	.3	.3	.3	.3	.3	.3			1.0		3
	249894	.3	.2	.2	.2	.2	.2		1.0			6
	249895	.6	.3	.6	.6	.6	.6		1.0			7
	249896	.3		.3	.3	.3	.3			1.0		3
	249897	1.0		1.0	1.0	1.0	1.0		1.0	1.0		2
	282442	.3		.3	.3	.3	.3		1.0			3
	364475	1.0		1.0	1.0	1.0	1.0	1.0	1.0			2
C. angolensis	--			1.0	1.0	1.0	1.0					1
C. dinteri	--	.8		1.0	1.0	1.0	.8		.8			5
C. sagittatus	282441	.9	.2	1.0	1.0	1.0	.2		.2	.1		9

Table 36. Continued.

Species	PI Number	Peroxidase Band Frequency at the Locus										
		Px_1	Px_2						Px_3			
			a'	a	b	c	d	d'	A	B	C	N*
C. dipsaceus	193498 236468	.9	.7	.9	.9	.9	.9			1.0		18
C. ficifolius	280231	.7			1.0	1.0	1.0	.7	.3	.7		7
C. asper	282443		1.0	1.0	1.0	.7						9
C. figarei	343699	.9		1.0	1.0	1.0	1.0	.9	1.0	1.0		7
	343700	.6		1.0	1.0	1.0	1.0	.4	1.0	.8		5
	343701	.4		.9	1.0	1.0	1.0	.6	1.0	.9		7
C. heptadactylus	282446	.8	1.0	1.0	1.0	1.0	.1		.3	.7	.3	38
C. africanus	203975	.2		1.0	1.0	1.0	1.0	1.0	.4		.2	5
	275571	.8	.2	.9	.9	1.0	.9	.3	.1	.2	.1	14
	374151	.1		1.0	1.0	1.0	1.0					7
C. leptodermis	282447	.8		.6	1.0	1.0	.8		.1		.8	31
C. myriocarpus	203977	.4	.4	1.0	1.0	1.0	1.0		.2	.4		5
	282449	.5	.4	1.0	1.0	1.0	1.0	.1	1.0	.4		8
	299568	.6	.4	.8	.9	.8	.8		1.0			16
	374153			1.0	1.0	1.0	1.0			1.0		1
C. melo	--	.7		.5	1.0	1.0	.5					>1000

Table 36. Continued.

Species	PI Number	Peroxidase Band Frequency at the Locus											N*
		Px_1	Px'_1	Px_2						Px_3			
				a'	a	b	c	d	d'	A	B	C	
C. prophetarum	179922	1.0			1.0	1.0	1.0	1.0					13
C. metuliferus	202681 292190		1.0	.9	.1	1.0	1.0	1.0					17
C. sativus	--					1.0	1.0	1.0					>100
C. hardwickii	215589					1.0	1.0	1.0					5
C. trigonus	271337					1.0	1.0	1.0					5
C. zeyheri	203974	.9		.5	1.0	1.0	1.0	.8	.3		.3	.4	8
	274036	1.0		.4	1.0	1.0	1.0	.8	.3			.6	9
	282450	.9		.5	1.0	1.0	1.0	.9	.3	.1		.6	8
	299569	.8		.1	1.0	1.0	1.0	.3				.4	10
	315212	.9		.9	1.0	1.0	1.0	1.0	.4			.3	8
	364473	.9		.7	1.0	1.0	1.0	.8	.1			.3	9
	273192	.8			1.0	1.0	1.0	.9	.6		.9	.1	14
	299570	.9		.1	1.0	1.0	1.0	1.0	.5		1.0	.4	10
	299572	.6			1.0	1.0	1.0	1.0	.6	.2	1.0	.6	5
	299571 A	.7		.3	1.0	1.0	1.0	1.0	.7	1.0	.3	.3	6
	299571 B	.9		.6	1.0	1.0	1.0	1.0	.8	1.0	.4	.3	8
	299571 C			.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.3	4

*N indicates the total number of samples run.

except for C. heptadactylus which has very anodically migrating isoperoxidases. Peroxidase alleles with multiple electrophoretic bands were also observed in maize (Brewbaker and Hasegawa, 1975) and Cucurbita pepo (Denna and Alexander, 1975). The maize alleles, however, were found to be under epigenetic control, while the Cucurbita pepo allele did not show developmental changes and was found to be codominant to an allele which produced only one band. The cluster of bands produced by the Px_2 alleles of C. leptodermis and C. melo (abc or bcd) was found to be under monogenic control, but additional Px_2 bands (a' and d') were observed in aged tissues. Differences at the Px_3 locus are apparent and are found within as well as between species (Table 36). Most diploid and all polyploid species have the Px_{3B} peroxidase. C. anguria var. longipes, C. figarei, and C. myriocarpus have Px_{3A} and C. africanus, C. leptodermis and C. zeyheri (2x) are characterized by a low or high frequency of the Px_{3C} allele. It seems to indicate that the annual C. africanus and C. leptodermis may have evolved from the perennial C. zeyheri (2x) and that C. figarei obtained the Px_{3A} peroxidase from C. anguria var. longipes or C. myriocarpus. However, since closely related species like C. africanus, C. leptodermis and C. myriocarpus or varieties of one species (C. anguria) have different Px_3 peroxidases, which are inherited as simple Mendelian codominant genes, the evolutionary significance of the Px_3 alleles is questionable. The low frequency of the Px_3 alleles in some species must be attributed to tissue or

developmental differences, environmental effects or slight differences in substrate specificity (o-dianisidine is known to visualize more Px_3 alleles than o-tolidine).

An increase in ploidy level was found to have little or no effect on the activity or electrophoretic mobility of peroxidase isozymes, which is in agreement with results obtained by Smith and Conklin (1975) and De Maggio and Lambrukus (1974). Although Bhatia et al. (1969) and Waines (1969) found a decrease in intraspecific variation with an increase in ploidy level, intraspecific variation was small in all Cucumis species.

Peroxidase patterns obtained after isoelectric focusing of the enzymes extracted from the cross-compatible Cucumis species show a high degree of similarity (Figure 33). Analysis of peroxidases with a high isoelectric point place C. africanus, C. dipsaceus, C. ficifolius, C. heptadactylus, C. myriocarpus, and C. zeyheri into one group and C. anguria, C. aculeatus, C. figarei and C. leptodermis in another. If other peroxidases are compared, however, the species will be distributed into slightly different groups. The peroxidase banding patterns obtained after isoelectric focusing show little relationship with the patterns obtained after electrophoresis. This may be the result of the use of o-tolidine on electrophoretic gels versus o-dianisidine on isoelectric focusing gels but more likely of the chelating action of the ampholines.

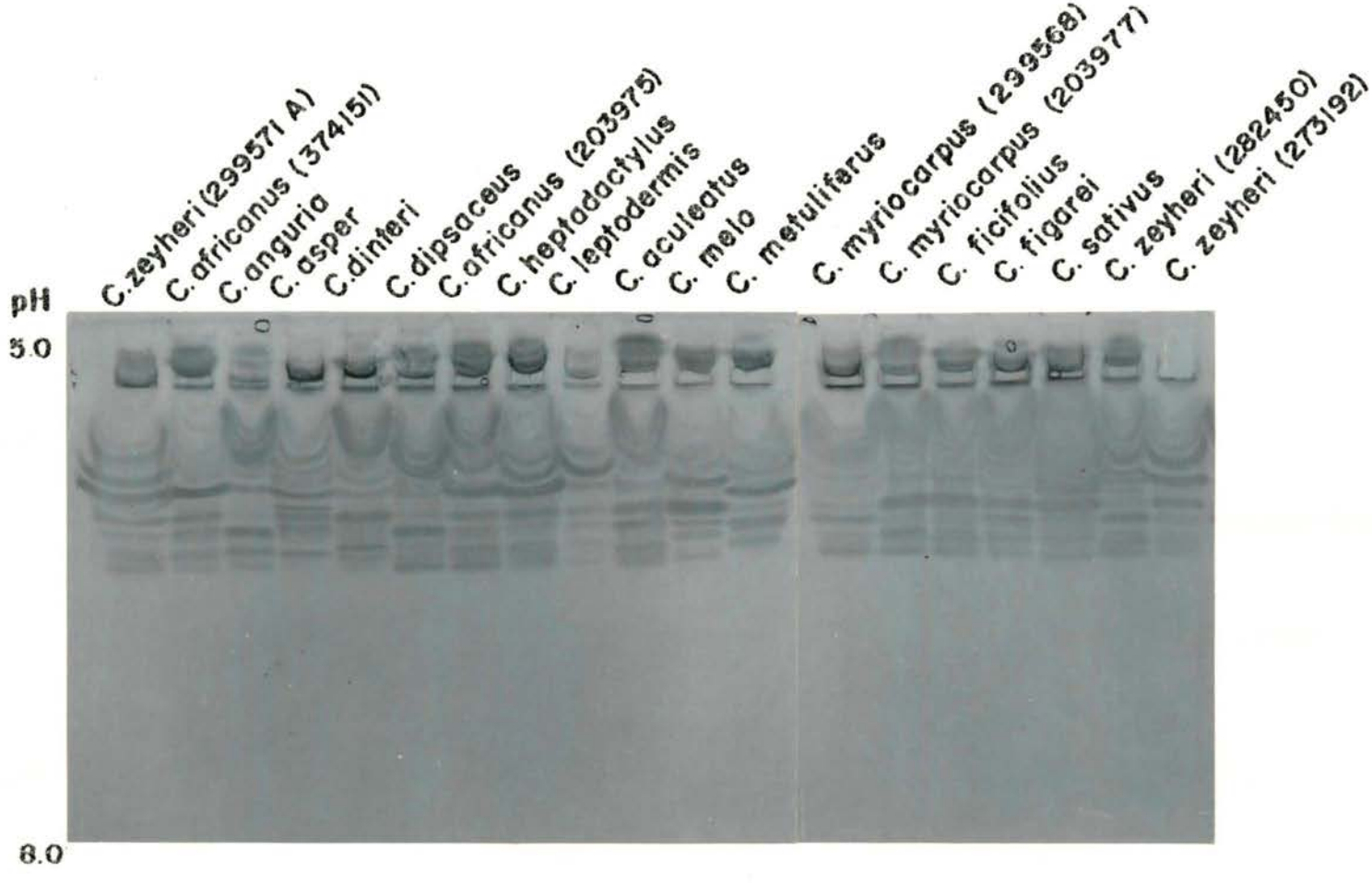

Figure 33. Peroxidase isoelectric focusing pattern of wild and cultivated Cucumis species.

While the GOT isozyme patterns of the cross-compatible species were similar, differences were observed in their esterase isoelectric focusing patterns (Figures 34, 35). The Ethiopian species seemed to have many high pI esterases in common and the South African species did likewise.

Chromotographic flavonoid patterns of the cross-compatible species (Brown et al., 1969) supported earlier conclusions about the close relationship between C. africanus, C. leptodermis, C. myriocarpus and the autotetraploid C. heptadactylus. C. zeyheri's flavonoid pattern however, showed more similarities with that of the Ethiopian species.

Similarity indices, calculated from the peroxidase and esterase isozyme banding patterns and flavonoid data of the species failed to show correlations. High similarity indices (varying mostly between 0.8-1.0) were obtained by comparing the peroxidase electrophoretic or isoelectric focusing patterns, but much lower values (varying mainly between 0.3-0.7) were found by comparing esterase isoelectric focusing patterns and even lower values (varying between 0 and 0.8) if flavonoid data were used. It is apparent that more interspecific differences can be detected by paper chromatography of flavonoid compounds than by electrophoresis or isoelectric focusing of the plant's enzymes. Electrophoresis and isoelectric focusing do detect only a small fraction (22%) of all possible mutations, only those that cause differences in the net electric charge of the protein molecule

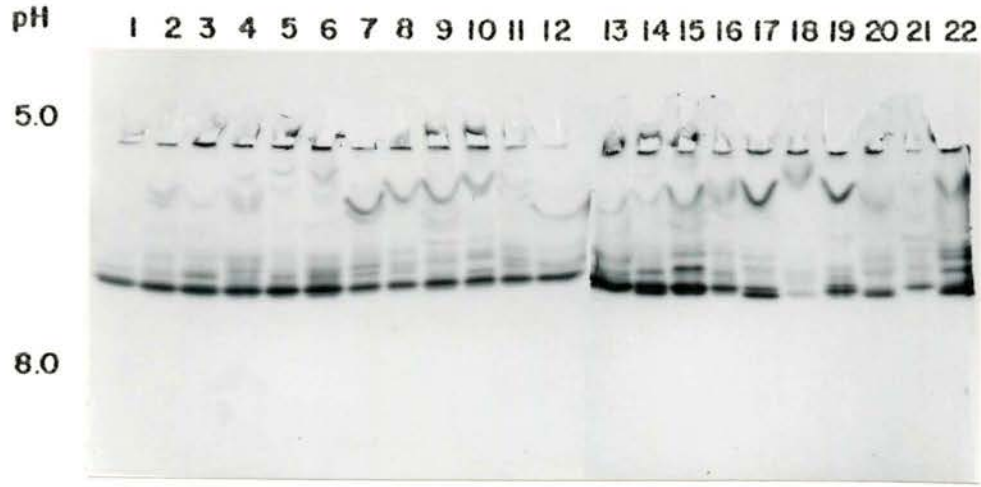

Figure 34. Esterase isoelectric focusing pattern of the wild and cultivated Cucumis species.

1. C. anguria (147065 x 282442)
2. C. anguria (364475)
3. C. dipsaceus
4. C. zeyheri (364473)
5. C. zeyheri (282450)
6. C. zeyheri (273192)
7. C. myriocarpus (299568)
8. C. myriocarpus (374153)
9. C. africanus (275571)
10. C. africanus (374151)
11. C. zeyheri (299571 C)
12. C. zeyheri (299571 A)
13. C. aculeatus (273648)
14. C. aculeatus (196844)
15. C. ficifolius
16. C. figarei
17. C. sativus
18. C. melo
19. C. heptadactylus
20. C. metuliferus
21. C. asper
22. C. dinteri

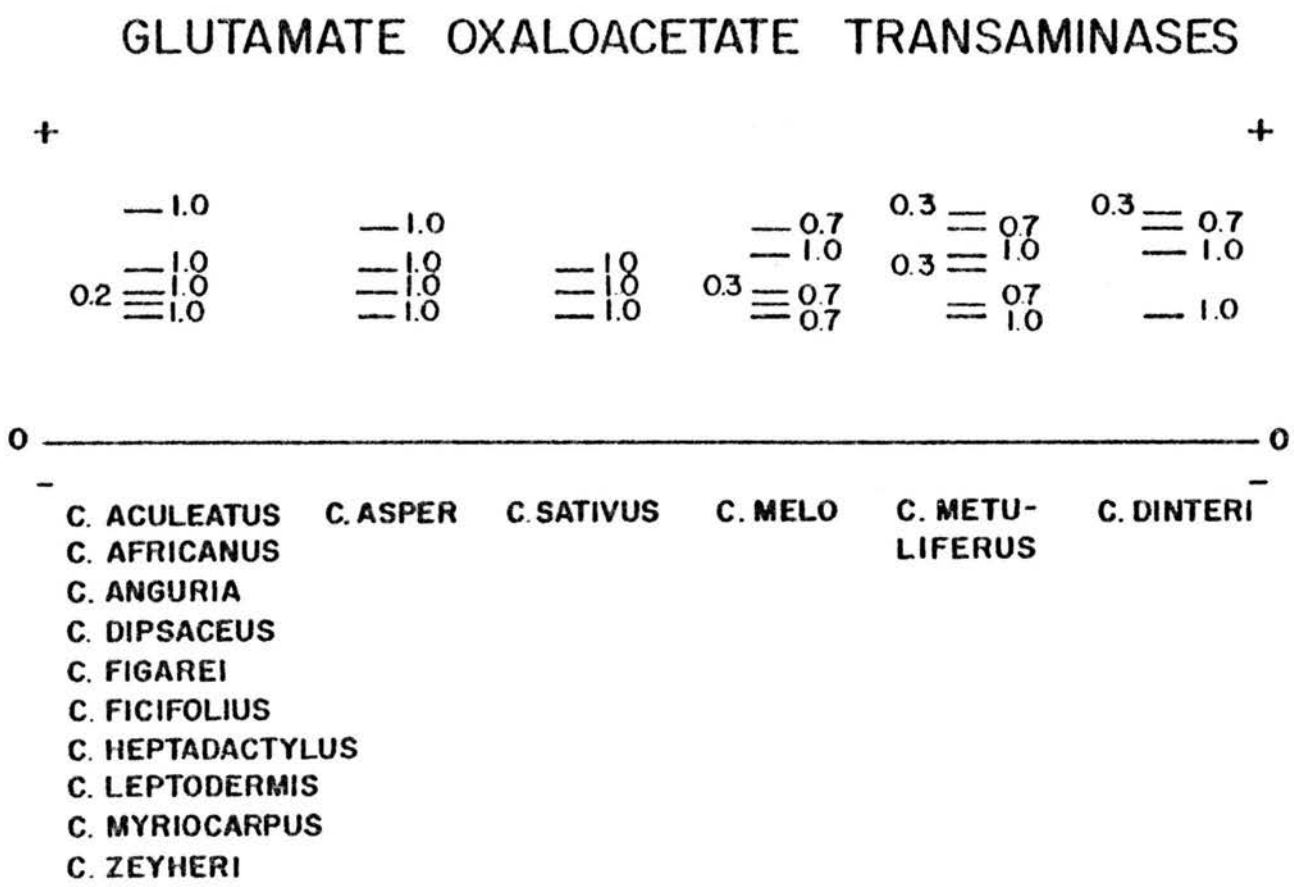

Figure 35. Glutamate oxaloacetate transaminase banding pattern of wild and cultivated Cucumis species.

(Powell, 1975). And similarity indices based on isozyme banding patterns may overestimate the evolutionary relationships between closely related species.

Cross-Incompatible Cucumis Species

The cross-incompatible group of species includes two of the economically important Cucumis species, the muskmelon or cantaloupe (C. melo) and cucumber (C. sativus), and three wild species, the annual C. metuliferus, and perennial C. asper and C. dinteri.

Cucumis melo and C. leptodermis show striking similarities in the migrational and genetic behavior of the Px_2 peroxidase alleles, and in their chromatographic flavonoid pattern (Brown et al., 1969). The GOT isozyme pattern of C. melo, however, shows many differences with that of the cross-compatible species (Figure 35). The overall similarity index calculated from all isozyme and flavonoid data (Table 37) point to C. leptodermis as the closest relative of C. melo. Their interspecific incompatibility barrier can be explained by their annual growth habit which resulted in the development of strong cytoplasmic or embryo-endosperm crossing barriers.

Cucumis sativus is different from all other Cucumis species because of its unusual low chromosome number (2n=14). It is characterized by the Px_{2B} peroxidase cluster and a flavonoid pattern different from that of all other Cucumis species. Antibodies developed against the Px_{2B} peroxidase cluster precipitated peroxidases of the

Table 37. Similarity index values calculated from the peroxidase (Figure 33; Table 36), esterase (Figure 34), GOT (Figure 35) and chromatographic flavonoid patterns (Brown et al., 1969).*

Species	C. anguria	C. asper	C. dinteri	C. leptodermis	C. melo	C. metuliferus	C. sativus
C. anguria							
C. asper	.47						
C. dinteri	.59	.47					
C. leptodermis	.56	.39	.45				
C. melo	.34	.36	.42	.51			
C. metuliferus	.28	.36	.34	.40	.31		
C. sativus	.33	.48	.31	.43	.34	.46	

*Similarity index = number of bands and/or compounds in common divided by the total number of bands and/or compounds.

Px_1, Px_2 and Px_3 loci of the cross-compatible and cross-incompatible Cucumis species (Figure 4). Precipitin patterns observed in the double diffusion plate tests are illustrated in Figure 36. Serologically identical antigen solutions will show precipitin bands arching into a chevron. Partial identity is indicated by the development of a spur growing towards the heterologous antigen (Crowle, 1973). However, spurs can be produced by use of disproportionate reactant quantities. Low concentrations of cucumber peroxidases and C. aculeatus, C. metuliferus, and C. melo peroxidases (Figure 36a, c and e), tested against the cucumber peroxidase antibodies resulted in the appearance of spurs. An increase in antigen concentration, however, indicated that the observed spurs were artifacts (Figure 36b, d and f) and that the antigenic determinant sites of peroxidase isozymes from the different Cucumis species are identical. The individual Px_2 b, c and d peroxidase isozymes of the cucumber also have identical antigenic determinant sites.

Cucumis sativus plants lack the Px_1 peroxidase found in all Cucumis species except C. asper and one GOT isozyme found in all cross-compatible Cucumis species (Figures 11 and 35). Table 37 indicates that C. sativus has more isozymes and flavonoids in common with C. asper and C. metuliferus than with the other species. The presence of 24 metacentric chromosomes in all diploid Cucumis species and longer chromosomes with secondary constrictions in C. sativus and the observed similarity of isozyme pattern suggest that

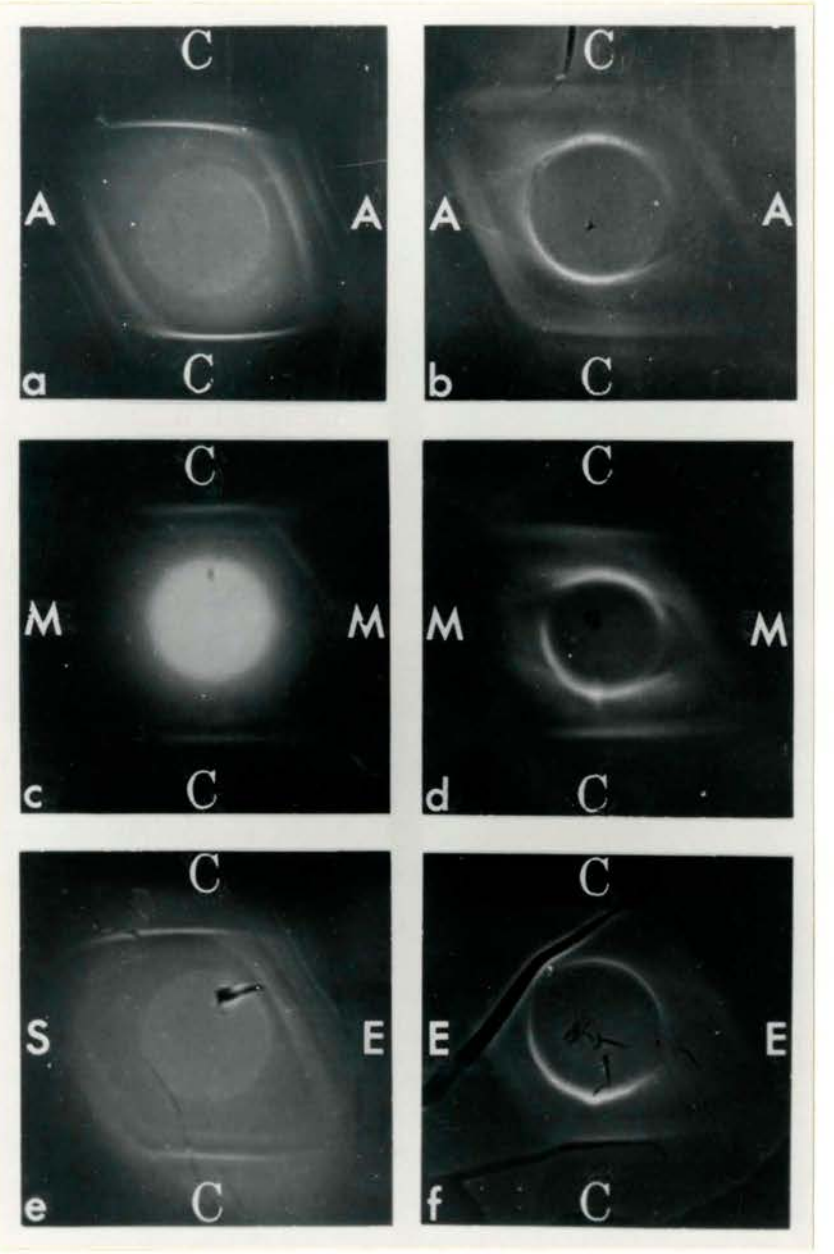

Figure 36. Immuno diffusion test plates with C. aculeatus (A), C. melo (M), C. metuliferus (E), C. sagittatus (S), and C. sativus (C) peroxidase antigens and serum II to the C. sativus peroxidases.

the C. sativus karyotype evolved from that of the other Cucumis species.

Cucumis metuliferus is distinctly different from all other species, which is expressed not only in its morphology but also in its isozyme and flavonoid patterns (section "Cucumis Species from South and South West Africa", subsection "Cucumis metuliferus" of this chapter). It appeared to have most isozymes and flavonoids in common with C. sativus.

The other cross-incompatible Cucumis species, C. asper and C. dinteri have woody whitish basal stems and a perennial growth habit, but while C. dinteri is monoecious, C. asper is dioecious. Table 37 indicates that the species have many isozymic variants in common but that C. dinteri is closer related to C. anguria.

Comparisons of the cross-incompatible species were based on similarity indices, calculated from isozyme and flavonoid patterns. The index will underestimate the evolutionary relationship of closely related Cucumis species, but overestimate that of distantly related Cucumis species since the presence of unusual (Px_1') or the absence of common (Px_1) bands will be weighted equally with the presence or absence of isozymic variants under monogenic control.

Although Lewontin (1974) found a high degree of genetic polymorphism and heterozygosity in animal species, and Allard et al. (1971) in plant species, most species in the genus Cucumis showed homozygous peroxidase loci. Polymorphism was observed at the Px_2

locus in C. anguria, C. heptadactylus, C. leptodermis and C. melo, and at the Px_3 locus in C. anguria and C. myriocarpus. While both Px_2 alleles were about equally distributed among C. melo accessions ($P_{Px_{2A}} = 0.51$), only one C. anguria accession had the Px_{2B} allele. Not only do the Cucumis species show a lack of polymorphism, they also have a low number of peroxidase loci as compared to other plant species (Scandalios, 1974; Brewbaker and Hasegawa, 1975; Rick et al., 1974). It must be related to their breeding system. Although the Cucumis species are monoecious or dioecious, the cultivated species are known to exhibit a low degree of inbreeding depression, which has been related to their small population size maintained not only under cultivation but also in nature and has resulted in inbreeding despite their floral mechanism favoring outcrossing (Whitaker and Davis, 1962).

SUMMARY AND CONCLUSIONS

The evolution of the wild and cultivated Cucumis species was studied by the determination and comparison of:

1. the seed, leaf, ovary, pollen and fruit morphology, growth habit and sex expression of the species;

2. the self-compatibility and intra- and inter-specific cross-compatibility levels;

3. the basic chromosome number; the pollen fertility and meiotic chromosome behavior of the polyploid species and of inter-specific hybrids;

4. the variation, inheritance and geographic distribution of electrophoretically detectable peroxidase isozymes;

5. the glutamate oxaloacetate transaminase (GOT) isozyme patterns obtained after polyacrylamide gel electrophoresis, and esterase and peroxidase patterns obtained upon isoelectric focusing of the enzymes extracted from two-three-week-old leaf and stem tissues;

6. the identity, partial identity or non-identity of the antigenic determinant sites of the peroxidase isozymes by double immuno diffusion tests in which Cucumis peroxidases are compared using antiserum to the C. sativus peroxidases;

7. similarity indices, calculated from isozyme and flavonoid (Brown et al., 1969) patterns.

The genus can be divided into a group of diploid cross-incompatible species which includes the cultivated C. melo (2n=24) and C. sativus (2n=14) and three South African species (C. asper, C. dinteri and C. metuliferus, with 2n=24 chromosomes), and a group of cross-compatible species distributed over South, East and West Africa.

The cross-compatible group contains three tetraploid (C. aculeatus, C. heptadactylus and C. zeyheri, with 2n=48 chromosomes), one hexaploid (C. figarei with 2n=72 chromosomes), five annual diploid (C. africanus, C. anguria, C. dipsaceus, C. leptodermis and C. myriocarpus, with 2n=24 chromosomes) and two perennial diploid (C. ficifolius and C. zeyheri, with 2n=24 chromosomes) species. The tetraploid C. aculeatus from Ethiopia and C. zeyheri from South Africa, and the hexaploid show only bivalent chromosome configurations at metaphase I, while the chromosomes of C. heptadactylus, which is indigenous to South Africa, form quadrivalent configurations. The autotetraploid nature of C. heptadactylus was also apparent in its pollen morphology. While the diploid and other tetraploid species develop 96-100% 3-porate pollen, 38% of the pollen mother cells of C. heptadactylus produced pollen with four or more pores. The hexaploid Cucumis species also showed an increase in the number of pores per pollen grain (26% 4-porate).

The annual Cucumis species showed the strongest, cytoplasmic or embryo-endosperm, incompatibility barriers with the perennial

species. While C. anguria developed vigorous F_1 plants in crosses with C. africanus and C. myriocarpus, the reciprocal crosses resulted in fruits lacking viable seeds. Fruits with inviable seeds were also produced between the annual species and the perennial diploids, while crosses with the allotetraploids were unsuccessful, but the reciprocal crosses resulted in vigorous F_1 plants. Meiotic chromosome studies of interspecific F_1 hybrids (Figure 37) indicated that the cross-compatible species have at least one genome in common and that chromosomal rearrangements have exerted an influence on the evolution of the Cucumis species. Although only one interspecific hybrid was found to be heterozygous for a translocation (C. dipsaceus x C. zeyheri) cryptic structural hybridity was indicated in other interspecific hybrids by a reduction in pollen fertility or in bivalent chromosome configurations. All polyploid species are perennial and have efficient vegetative reproduction systems.

Most species in the genus Cucumis displayed a homozygous peroxidase banding pattern characterized by peroxidases at three independently segregating genetic loci (Px_1, Px_2 and Px_3). Px_1, the most anodically migrating peroxidase is absent in C. asper and C. sativus and displaced in C. metuliferus. The Px_3 peroxidases, the least anodically migrating, are lacking in C. asper, C. melo, C. metuliferus and C. sativus. Peroxidase polymorphism was observed at the Px_2 locus in C. anguria var. longipes, C. heptadactylus, C. leptodermis and C. melo, and at the Px_3 locus

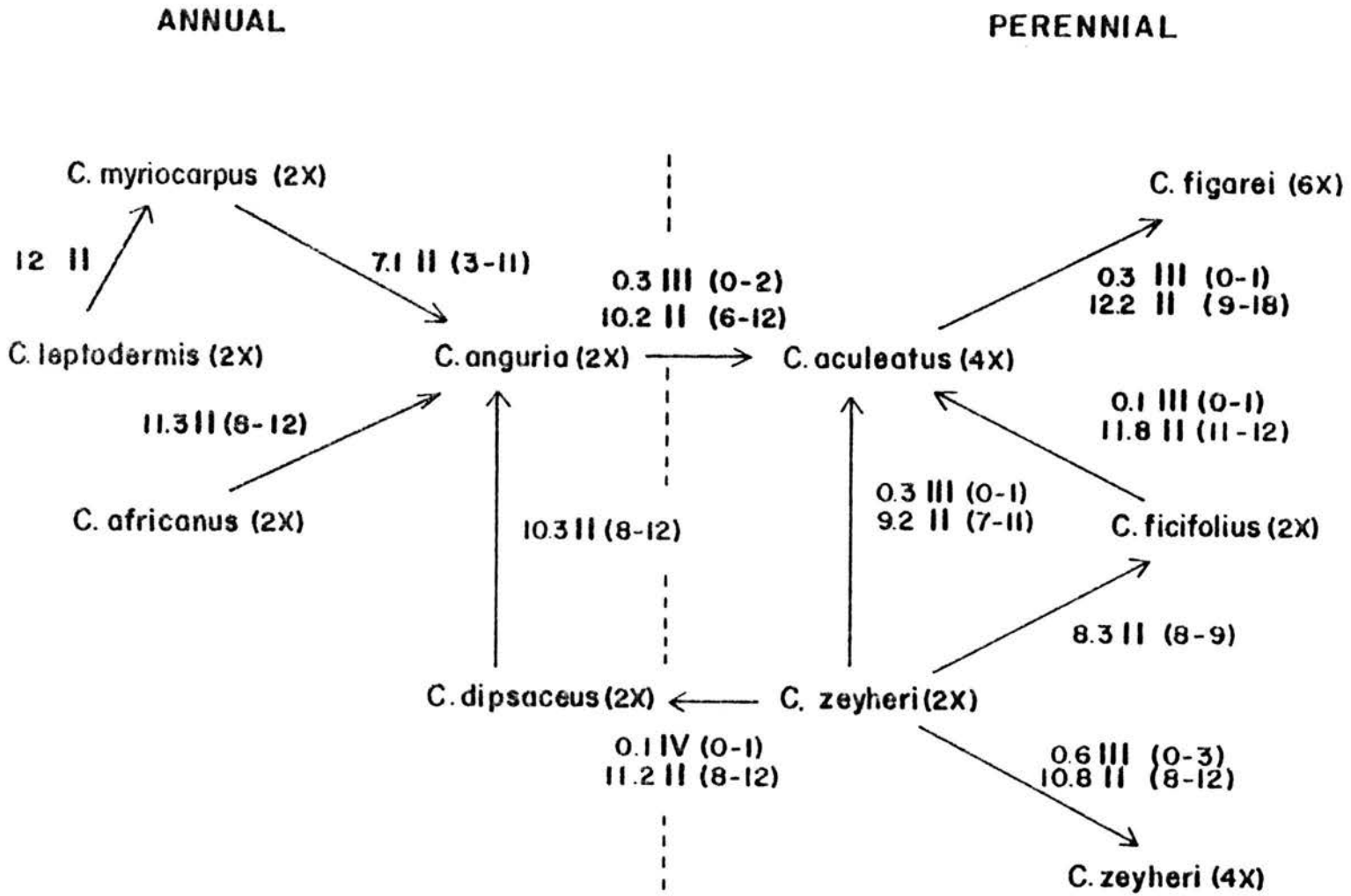

Figure 37. Average number and range (between parentheses) of bi-, tri-, and quadrivalent chromosome configurations of interspecific F_1 hybrids between the cross-compatible Cucumis species. Arrow indicates the direction of pollen transfer.

in C. anguria and C. myriocarpus. The variant Px_2 alleles of C. melo and C. leptodermis, which are represented by a cluster of three bands, are codominantly inherited. The Px_3 alleles of C. myriocarpus are also codominant.

Some species showed unique peroxidase banding patterns (C. anguria var. anguria, C. asper, C. figarei, C. heptadactylus, C. leptodermis, C. melo, C. metuliferus and C. sativus), but similarities were apparent. This was also indicated by the immunological studies which showed that the antiserum to the cucumber peroxidases (Px_{2B}) precipitated peroxidases of the Px_1, Px_2 and Px_3 loci of the cross-compatible and cross-incompatible Cucumis species and that the antigenic determinant sites of these peroxidases were identical.

Muskmelon (C. melo) Plant Introductions showed a geographic variation in their Px_2 allele frequencies, which could not be correlated with variation in morphological characters. It could have been the result of slightly different selection pressures on the individual alleles, since disease resistant C. melo accessions (except mildew and Macrosporium resistant PI's) were found to have a high frequency for the more anodically migrating Px_{2A} allele, and since the distribution of the alleles appeared to be nonrandom. However, the establishment of small populations in some areas may have resulted in inbreeding despite the floral mechanism favoring outcrossing.

The cross-incompatible species could be distinguished from each other and from the group of cross-compatible species by their

GOT banding pattern. The differences were less significant in their esterase and peroxidase isoelectric focusing patterns, although unique species-specific patterns were observed. An increase in ploidy level was found to have little or no effect on the activity or electrophoretic mobility of the peroxidase, GOT, and esterase isozymes.

While high similarity index values were obtained when the peroxidase isozyme patterns of the Cucumis species were compared, lower values were found when esterase bands were used and even lower values when their chromatographic flavonoid patterns were compared.

The results of the biochemical, cytological, morphological and hybridization studies suggest the following phylogenetic relationships:

1. C. melo (the muskmelon or cantaloupe) is closer related to a group of South African species, C. africanus, C. leptodermis and C. myriocarpus than to other Cucumis species.

2. C. sativus, the cucumber, is closer related to C. asper and C. metuliferus than to other Cucumis species. C. hardwickii and C. trignous are conspecific to C. sativus. Their karyotype with 2n=14 chromosomes probably evolved from that of the other Cucumis species with 2n=24 chromosomes.

3. C. anguria var. anguria (the West India gherkin) descended from Ethiopian accessions, which are conspecific to the South African C. anguria var. longipes.

4. C. africanus, C. leptodermis and C. myriocarpus can be considered as subspecies, which are closely related to the autotetraploid C. heptadactylus.

5. C. ficifolius is one of the ancestors of the allotetraploid C. aculeatus.

6. The diploid C. zeyheri is one of the progenitors of the allotetraploid C. zeyheri.

7. C. aculeatus has two genomes in common with the hexaploid C. figarei.

8. C. dinteri, which is conspecific to C. angolensis and C. sagittatus, is closer related to C. anguria than to any of the other cross-incompatible species.

Phytogeogeographic and host-parasite relationships of the wild Cucumis species point to South Africa as the primary gene center of the genus Cucumis. Only species from the successful cross-compatible group moved northward to Ethiopia where a secondary gene center was established and to Nigeria.

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APPENDIX

Appendix Table 1. Distribution of Cucumis melo peroxidase genotypes.

Country	Peroxidase Genotype			N	Gene Freq.	X^2
	$Px_{2A}Px_{2A}$	$Px_{2A}Px_{2B}$	$Px_{2B}Px_{2B}$		$P_{Px_{2A}}$	
Afghanistan	69	98	50	217	.54	3.32
Argentina		1		1		
Asia		1		1		
Australia	1			1		
Austria	1			1		
Belgium	1			1		
Burma	1	1		2		
Africa		1		1		
Canada	9	34	4	47	.55	1.06
China	1	4	6	11	.27	4.55*
Ecuador	1			1		
Egypt	3	6	5	14	.43	.58
El Salvador	1	4	1	6		
Ethiopia	1			1		
Finland	1			1		
France	2	1		3		
Greece	1	3	2	6		
Hungary	1		2	3		
India	101	141	41	283	.61	25.44**
Iran	23	49	6	78	.61	7.42**
Israel	3	3	1	7		
Japan	1	12	3	16	.44	.50
Korea	3			3		
Lebanon	1			1		
Mexico		2	2	4		
Morocco			1	1		
Netherlands	1			1		
Nigeria			1	1		
Pakistan		2		2		
Peru		1	1	2		
Portugal		1		1		
Saudi Arabia		3		3		
Senegal	1			1		
South Africa	1	2		3		
Spain	3	7		10	.65	1.80
Syria	1	3	7	11	.23	3.28
Switzerland		1		1		
Taiwan	2			2		
Thailand	1			1		
Turkey	37	74	114	225	.33	26.36**
U.S.A.	3	3	5	11	.41	.72
U.S.S.R.	2	8	9	19	.32	2.58
West Pakistan	1	1	1	3		
U.S. Varieties	40	75	40	155	.50	0
Total	319	542	302	1163	.51	0.50

*Significant at the .05 level of probability.

**Significant at the .01 level of probability.

Appendix Table 2. Fruit shape index of Cucumis melo Plant Introductions from various countries.

Country	Fruit Length (cm)	Fruit Width (cm)	L/W Index	L/W CV	N
Afghanistan	22.6	15.1	1.53	35.6	207
China	21.2	15.0	1.44	24.1	9
Egypt	23.8	12.4	1.97	45.8	14
India	21.9	14.2	1.68	67.3	236
Iran	24.4	15.6	1.64	61.3	72
Japan	16.6	12.2	1.38	41.1	18
Syria	26.4	13.6	2.14	82.9	8
Turkey	20.9	13.7	1.64	61.5	150
U.S.S.R.	21.1	14.8	1.44	50.0	23

Appendix Table 3. Genotype and gene frequency of round, oblong and elongate fruits of Cucumis melo Plant Introductions from various countries.

	Fruit Shape Genotype Frequency					
Country	Round L/W=1.0	Oblong $1 < L/W < 2$	Elongate $L/W \geq 2$	N	Gene Freq.	X^2
Afghanistan	.13	.76	.11	204	.51	.25
China		1.00		7	.50	0
Egypt	.07	.57	.36	14	.36	2.29
India	.20	.56	.24	214	.48	.60
Iran	.26	.54	.20	69	.53	.46
Japan	.44	.44	.12	16	.66	3.13
Syria	.29	.29	.43	7	.44	.29
Turkey	.22	.59	.19	143	.52	.35
U.S.S.R.	.33	.52	.14	21	.59	1.52
Total	.20	.62	.18	695	.51	

Appendix Table 4. Fruit color of Cucumis melo Plant Introductions from various countries.

Country	Fruit Color			N
	% Yellow	% Orange	% Green	
Afghanistan	60.4	33.8	3.6	222
China	44.4	55.6		9
Egypt	80.0		10.0	10
India	58.2	32.9	8.5	213
Iran	61.0	26.0	8.0	77
Syria	77.8	11.1	11.1	9
Turkey	57.3	15.3	26.8	157
U.S.S.R.	83.3	16.7		18
Japan	80.0	6.7	6.7	15
Total	60.4	27.3	10.6	

Appendix Table 5. Fruit surface of Cucumis Melo Plant Introductions from various countries.

Country	Fruit Surface				N
	% Smooth	% Furrowed	% Netted	% Wrinkled	
Afghanistan	38.3	33.0	17.0	9.6	188
China	44.4	22.2	33.3		9
Egypt	40.0	40.0	10.0	10.0	10
India	38.5	37.7	21.0	1.6	252
Iran	44.2	31.2	22.1	2.6	77
Syria	37.5	50.0	12.5		8
Turkey	24.8	17.4	14.9	41.4	161
U.S.S.R.	63.2	26.3	10.5	12.3	19
Japan	55.0	35.0	0.5		20
Total	37.3	31.1	18.1	11.9	

Appendix Table 6. Cucumis melo Plant Introductions from various countries with resistance to downy mildew, powdery mildew, gummy stem blight, anthracnose, and Macrosporium.

Country	Downy Mildew		Powdery Mildew		Gummy Stem Blight		Anthracnose		Macrosporium	
	%	(N)*	%	(N)	%	(N)	%	(N)	%	(N)
Egypt	37.5	(8)	0	(7)	0	(1)	0	(7)	0	(0)
India	43.6	(101)	24.5	(94)	3.4	(29)	10.7	(56)	28.6	(28)
Syria	28.6	(7)	0	(5)	0	(1)	0	(2)	0	(3)
Turkey	17.1	(129)	0.8	(119)	0	(11)	2.1	(21)	15.1	(73)

*N indicates the total number of PI's tested.