Gene Flow in *Cucurbita*

by

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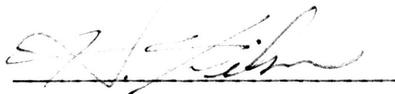
Approved by,



Dr. Hugh D. Wilson

This work is dedicated to Kitt Kleopfer
for her friendship and assistance.

ABSTRACT

Gene Flow in *Cucurbita*

Cucurbita pepo and *Cucurbita texana* when artificially crossed produce fully fertile hybrids. Thus interspecific genetic exchange is possible. An attempt was made to monitor actual gene flow between these two taxa under both agricultural and natural conditions. Two synthetic populations were established for this purpose using experimental plants that were genetically marked by isozyme phenotype. At the end of the growing season progeny were electrophoresed. The resulting data quantified the event of genetic exchange between taxa, revealed the source of foreign genetic material, and thus established the distance over which gene flow occurred. It can be concluded from the data that since genetic exchange does indeed take place between the *C. pepo* complex and *C. texana*, their classification as two distinct and separate species is in question. The data also indicate that the previously published distance requirement for genetic isolation within the *Cucurbita* is incorrect.

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I. INTRODUCTION

Cucurbita is a genus composed of the various gourds, squashes and pumpkins, all of which are indigenous to the Americas. American "farmers" have been using certain species of the genus as both a staple food and a tool for more than 5,000 years (Whitaker et al. 1957). Of the twenty-seven species within the Cucurbita, only five have been domesticated. Previous work in relation to the phylogeny of Cucurbita species has centered on the use of artificial interspecific crosses to determine their placement within a phylogenetic framework based on genetic barriers to successful reproduction. Such artificial hybridization studies provided the basis for suggesting possible genetic links between species, and thus a possible path for evolutionary progression as it occurred within the genus (Whitaker et al. 1975).

Cucurbita texana (Scheele) Gray, the native "Texas gourd" and Cucurbita pepo L. var. ovifera Alef., the domesticated "ornamental" or "yellow-flowered" gourd, have long been identified as two closely related taxa. The difficulty in assessing their taxonomic relationship is due in large part to their multiplicity of morphological variations (Bailey 1937). Cucurbita texana was originally described by Scheele in 1848 as Tristemon texana based on a collection by Lindheimer, and was later transfered to the genus Cucurbita by Asa Gray. On making this change, Gray remarked that the "fruit is just that of Cucurbita ovifera, of which our plant may possibly be only a naturalized variety," (Gray 1850). This position was supported by Coulter, (Coulter 1891). However, Gray later wrote that the plant had the appearance of being native; "At least this is the opinion of Mr. Lindheimer and Mr. Charles Wright (both well

known students of the Texas flora) two good judges. The later informs us that, from the stations and localities in which alone it is met with, he could not so feel it to be other than an indigenous plant," (Gray 1857). Several other students of *Cucurbita*; Bailey (1930), Erwin (1938), and Correll and Johnston (1970), upon examining the plant growing wild, also concur with this opinion.

If one accepts the current biological definition of a species as a group of actually or potentially interbreeding organisms reproductively isolated from other such groups, then *C. texana* and *C. pepo* should obviously not possess the ability to interbreed. However, artificial interspecific crosses made between *C. texana* and *C. pepo* in an effort to determine the evolutionary history of the genus resulted in the formation of fully fertile hybrids (Whitaker and Bemis 1965). Thus, the potential for gene flow between populations of both species exists. Demonstration, however, of the presence or absence of actual interspecific gene flow would clarify this taxonomic relationship within the *Cucurbita*. It should be noted, though, that the biological definition is presently not accepted by some biologists, who maintain that, because of the nature of higher plant genetic systems, empirical definitions of plant species as natural units of evolution cannot be made, for such units are either nonexistant or can only be abstractly conceived (Levin 1979).

It is not the author's point to debate this issue, because regardless of whether or not a plant species can meet certain stringent quantitative qualifications, it is necessary to have a basic evolutionary and taxonomic unit which incorporates the parameter of genetic exchange into its definition, and so the biological definition is maintained here.

One of the primary objectives from the onset of this research was, therefore, to determine if genetic exchange can occur between *C. texana* and *C. pepo* under natural conditions, incorporating natural pollen vectors. Some means of monitoring gene flow between *Cucurbita* populations would be necessary to determine if interspecific hybridization was occuring, and developing this procedure was also a specific goal of the project. Because gene flow is such a critical aspect of plant population biology, an accurate method of quantifying the event would certainly have many applications. Prior work, however, has been limited to observation of pollen flow and seed dispersal rather than actual genetic exchange, or has incorporated the use of dominant/recessive genetic markers affecting the gross morphology of only the recessive parent's hybrid progeny (Handel 1982). The use of such markers is limited to the monitoring of unidirectional gene flow and may possibly invite selective pressure against such morphologically modified cultivars. Therefore the development of isozyme analysis as a means of demonstrating actual gene flow among plant populations was, of necessity, an integral part of the research.

II. MATERIALS AND METHODS

Taxa Incorporated

The genus *Cucurbita* is a well defined group within the Cucurbitaceae, all of its species being monoecious. The flowers are solitary, relatively large and showy. Species of *Cucurbita*, particularly the cultivated species, are not easily distinguished by any particular morphological trait, and there is a tendency toward parallel variation and phenotypic plasticity. The genus is assumed to be of relatively recent phylogenetic origin (Whitaker and Bemis 1975). Evidence to this effect can be found not only in the vague morphological differences of its members, but also in the fact that all *Cucurbita* species have the same number of chromosomes (2n = 40) (Whitaker and Davis 1962). To further support this idea of recent origin, the use of SEM analysis has recently revealed uniformity in size and shape of mature pollen grains from 18 species of *Cucurbita* (Andres 1983). Also, one cannot ignore the significance of the fact that no single species of *Cucurbita* or group of species is reproductively isolated from all other species of the genus in terms of artificial interspecific crossing (Hurd et al. 1971).

Cucurbita pepo is a large, coarse, polymorphic species, with variation occuring in both vegetative and reproductive characters. Only cultivated varieties are known, with no record of *C. pepo* ever becoming naturalized. *C. pepo* includes the numerous cultivars of summer and winter squashes, pumpkins, and ornamental gourds. There have been several attempts to delimit the group at the subspecific level. Classification systems for the species have included anywhere from two to eight varieties. For the purposes of this experiment, *C. pepo* has been

artificially divided into four subspecific groups based primarily on the use of the fruits by man (Bailey 1929, 1949; Purseglove 1974). Three of these four subspecific entities were directly incorporated into the experimental procedure: C. pepo var. ovifera (L.) Alef. (CPO), C. pepo var. melopepo (L.) Alef. (CPML), and C. pepo var. medullosa Alef. (CPMD).

CPO are the ornamental gourds which are often attractively shaped and colored. Their fruits are typically characterized by a hard pericarp with very little flesh, the flesh often being bitter and thus inedible due to the presence of a class of organic compounds appropriately named cucurbitacins (Whitaker and Bemis 1975). The plants are slender, long-running or climbing vines with tendrils. A cultivar producing yellow, warty fruits was used. CPML are most often recognized as summer squashes or squashes which are consumed in the immature state. The particular cultivar of CPML used throughout this study was the early summer crookneck squash. CPMD is a somewhat homogeneous group recognized as the Italian marrows. All cultivars from this group have elongated fruits, and the black zucchini type was selected for the procedure. Both CPML and CPMD plants have compressed internodes with abortive tendrils and are commonly referred to as bush squash.

The second species of interest used in this study, C. texana (Scheele) Gray, abbreviated CT, is known commonly as the Texas gourd. This wild, spontaneously occuring gourd is similar to CPO in having relatively small, hard-shelled, bitter tasting fruits. The species has long been thought endemic to Texas, occuring "in debris and piles of driftwood, often climbing into trees, along several rivers, especially the Guadalupe, that drains the Edwards Plateau in central Texas" (Correll and

Johnston 1970). Populations in Texas are uncommon and localized. Isolated occurrences of spontaneous gourd populations in the southern to midwestern U.S. have variously been treated as CPO or CT.

Isozyme Labeling

Because of the nature of the project, which involved an assessment of the possible genetic exchange between various taxa within the *Cucurbita*, a mechanism for monitoring actual gene flow among plant populations under natural conditions had to be established. The most suitable means for accomplishing a quantitative inquiry into the actual gene flow problem proved to be a process of isozyme labeling of the experimental plants. Before the advent of molecular technique in population genetics, "the traditional methods of analysing genetic variation were stymied by the impossibility of equating phenotypes with genotypes" (Gottlieb 1971). Today, through the use of gel electrophoresis, variation within a population can be examined quickly and efficiently. The use of gel electrophoresis is also unique in that it allows the visualization and quantification of variation at the simplest level of genetic expression: that of the primary structure of proteins, or simply, changes in the amino acid composition of such proteins. The technique is based upon the fact that such changes in protein structure are the direct result of variation within the nucleotide sequence of the structural genes coding for the product. Changes in the amino acid composition of a protein, whether it be a deletion, addition, or substitution, can alter the net electrostatic charge on that protein and thus overtly affect the migration of that protein through an electric field. Such different molecular forms of the same enzyme are termed isozymes (Markert and

Moller 1959). Upon electrophoresis and subsequent staining of a gel for a particular enzyme system, different isozymes will appear as distinct phenotypic banding patterns on the gel. Seed stocks for the three varieties of C. pepo and C. texana to be used in the study were selected and planted in the laboratory. Cotyledon material from the seedlings of each taxon were electrophoresed and stained for three enzyme systems: PGI, GOT, and PER (Fig. 1). Only those seedlings exhibiting selected phenotypes in all three enzyme systems were chosen for planting in the field. Phenotypes useful as genetic markers for each taxon were selected so as to fascilitate easy recognition of outcrossing if it occured.

Establishment of Populations

In order to monitor genetic exchange as it occurs among populations of Cucurbita, land was obtained from the agricultural branch of Texas A&M University at A&M Farms in the Brazos Valley of central Texas. Under the assumption that the published limit of Cucurbita gene flow was accurate at 400 meters maximum isolation distance necessary in order to insure genetic purity of seed (Kernick 1961), two experimental populations were estabished in the Spring of 1982 to assess the potential for interspecific crossing under different environmental conditions (Fig. 2). Stations 1 through 5 were established in such a way as to determine the potential for hybridization under agricultural conditions. CPML was grown at station 5 in a garden-like arrangement consisting of a single 12 meter row, while stations 1 through 4 represented isolated occurrences of CT along a fence row.

The second experimental population, stations 6 through 15, was an attempt to portray a more natural occurrence of the two species in the

wild condition. Station 14 was planted in such a manner as to represent a small 6x6 meter "patch" of CPO with CT stations variously located along the banks of the Brazos River, the flood-plain being representative of its natural habitat.

Flower Development and Pollinator Activity

Frequent visits to the site were necessary in order to insure that each genetically marked plant became established, and once flowering began in late June, daily visits were necessary in order to record data pertinent to the study of the gene flow problem. Information such as the number of pistillate and staminate flowers per plant had to be collected on a daily basis due to the fact that the flowers of *Cucurbita* open and wither in a single day (Hurd et al. 1971). Pistillate flowers were marked on the day of their opening for later identification of fruits in the case of fruit set so that the date of pollination would be known.

The activity of certain bees was noted and various potential pollinators found in association with the different taxa were caught for identification. When circumstances allowed, bees were marked to permit monitoring of movement within the study area. Various colors of liquid paper were dabbed on their thorax to identify the bees with a particular station.

Electrophoretic Procedure

The various fruits were allowed to mature on the plants and then harvested in middle to late summer. The picked fruits were dried, and the seeds extracted. The dried seeds were then germinated in an incubator and sown to produce the progeny generation in the laboratory. Previous work had shown that as soon as the young plants put forth their

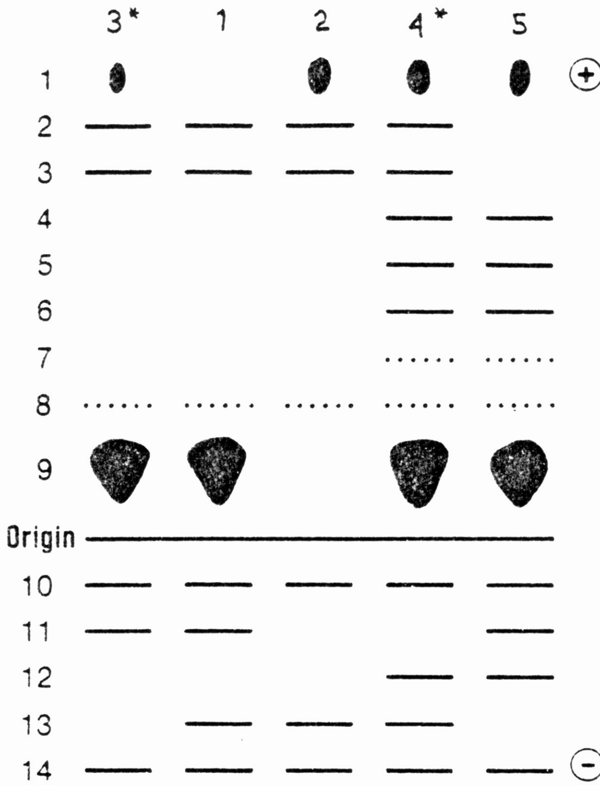
first true leaves they were at the proper age for electrophoresis.

The night before electrophoresis was to be done, starch gels were prepared. Standard methods for horizontal starch gel electrophoresis in a PK buffer system were used (Appendix I & II). After the starch gels were poured and the surface of each had solidified, they were covered with cellophane and allowed to cool overnight. The following morning, cotyledons from the progeny seedlings were picked and placed in homogenizing blocks. The plant material was then homogenized by grinding it with a drill press fitted with a blunt acrylic bit designed to fit snugly into the individual sample wells of the homogenizing block. The resulting crude homogenate was immediately absorbed onto 2x10 mm wicks made of Whatman #3 chromatography paper. Once saturated, the wicks were then loaded into the gel at the origin, a slice made in the gel 6 cm from one end. Interior gel mold dimensions were 14.5x10x.7 cm . Thirty wicks, and thus thirty individual samples, could be evenly spaced along the width of the gel, separated by at least 2 mm to avoid cross contamination, improve band resolution and fascilitate easy scoring.

Once the wicks were loaded, the gel was re-covered with the cellophane and electrophoresed at 200V and 50mA for thirty minutes, after which the wicks were removed. The electrophoresis then continued at 200V until the borate front of the discontinuous tris-citrate or PK buffer system (a modification of Poulik 1957) moved the 6 cm necessary to cross the origin and progress toward the anodal end of the gel. Immediately after the front had crossed the origin, the voltage was increased to 250 V at 50mA maximum. All electrophoresis was done under refridgeration at $4^{o}C$. After approximately six hours of electric field exposure, the gels

were removed for slicing and staining. Three horizontal slices of approximately 2 mm thickness were taken from each gel electrophoresed, and each was stained for one of the three enzyme systems used to genetically mark the experimental plants in the field (Appx. III). The enzyme systems examined were phosphoglucose isomerase (PGI), E.C. 5.3.1.9; glutamate-oxaloacetate transaminase (GOT), E.C. 2.6.1.1; and peroxidase (PER), E.C. 1.11.1.7. Only anodal activity was used to determine the enzymatic phenotypes of these progeny plants. By comparison of the progeny phenotype in each enzyme system with that of the known egg parent, the occurrence of gene flow could easily be recognized, the pollen parent's taxon identified, and the event readily quantified.

Fig. 1. Resulting phenotypes expressed by differential migration of isozymes in the PGI, GOT, and PER enzyme systems using starch gel electrophoresis. * Denotes a heterozygous phenotype.


PER:
3*
1
2
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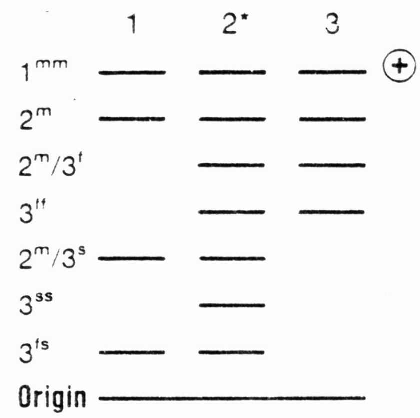
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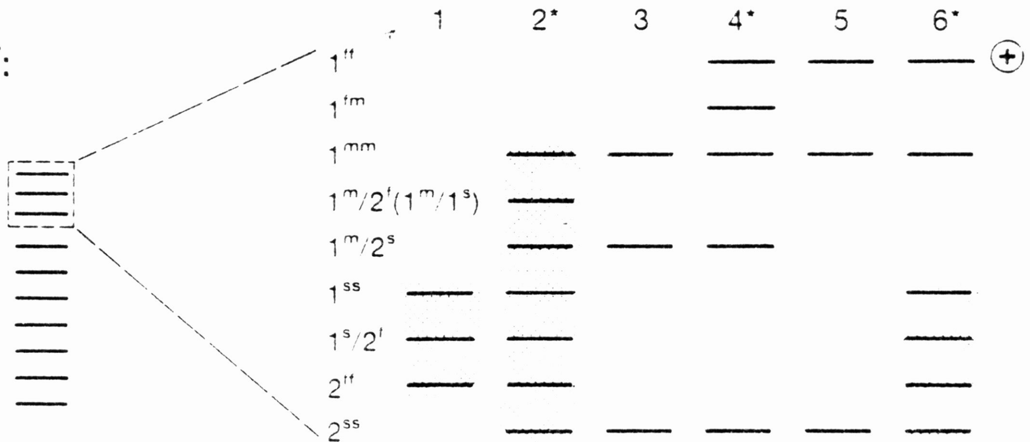
Origin


GOT:
1
2*
3
4*
5
6*
1ff
1fm
1mm
1m/2f(1m/1s)
1m/2s
1ss
1s/2f
2ff
2ss

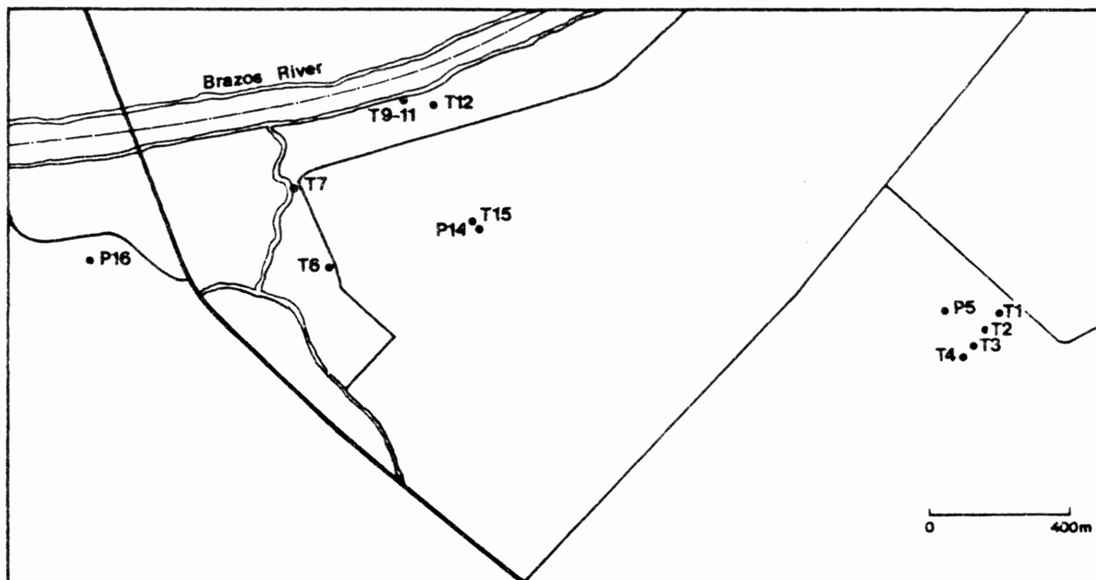


Fig 2

Diagram showing distribution of the two experimental populations on land of Texas A&M Farms. "Agricultural" population is depicted by stations 1 through 5. "Natural" population is represented by stations 6 through 15.

P = Cucurbita pepo

T = Cucurbita texana

Note: 400m scale representative of previously published isolation requirement for Cucurbita.

III. RESULTS

Bees and other potential pollinators found in association with the experimental plants were caught and identified (Table 1). Based on the results of this identification, the wild bee, *Xenoglossa strenua*, was determined to be the primary pollinator working the taxa of interest within the study area. *Xenoglossa* far outnumbered the other wild bee, *Peponapis*, found in the study area as well as *Apis mellifera*, the local honeybee. The "squash bees", *Xenoglossa* and *Peponapis*, commonly overnight in the wilted flowers of *Cucurbita* (Linsley 1960), and several instances of finding *Xenoglossa* in this circumstance occurred.

Because of the high frequency of *Xenoglossa* sightings among the experimental plants, an attempt was made to monitor their movement in and between stations (Table 2). According to the table, *Xenoglossa* tended to remain associated with a particular area as seen in the high number of instances a marked bee was re-caught at the same station it was originally tagged. Much more infrequently, however, instances of bees moving between stations were monitored. For example, bees marked at stations along the fencerow (1-4) were seen at station 5, and vice-versa. *Xenoglossa* was also observed to have travelled from station 5 to station 14 and from 14 to 7. From such observations of pollinator movements, the potential for interspecific gene flow was established in the "agricultural" and "natural" populations of *Cucurbita*. Also, evidence of possible gene flow between these synthetic populations at distances far in excess of previously published limits was revealed.

The number of staminate flowers produced by plants representing the three individual taxa used in the study was recorded on a daily basis

over a two month period in the summer of 1982 (Fig 3). This flowering phenology represents the total contribution by each taxon to pollen and nectar availability on a temporal scale. Each taxon exhibited a peak of flowering activity at a different time in relation to the others. CPML led the way by peaking early in July. CT, however, exhibited a more obtuse peak which extended through the middle third of the month. After the maximal flowering activity of CT, it was finally CPO's turn at the top, this taxon reaching a sharp pinnacle of flowering near the end of July. The information gained from this flowering phenology was used in conjunction with the results of isozyme data to give an indication of conditions necessary or beneficial for interspecific gene flow to occur.

The net result of screening the progeny of the experimental plants for isozyme phenotype is shown in Table 3, a condensed version of the original electrophoretic data (Appx. IV). CPO at station 14 was set apart from the other taxa by exclusively exhibiting a type 3 PGI phenotype. CPML (with the exception of CPML-2) and CT, on the other hand, expressed exclusively type 1 PGI. The occurrence of type 2 PGI in progeny of CPO - 3, 4, and 5 is illustrative of the heterozygous condition and the result of a cross with the type 1 PGI of CPML or CT. To resolve the problem of which taxon was the pollen parent, the GOT enzyme system must be examined. CPO was (with the exception of CPO-1) genetically marked with a GOT 3 phenotype as was CPML. The occurrence of a type 2 GOT in either CPO or CPML progeny indicates the heterozygous condition and is the result of an outcross from the type 1 GOT unique to CT. It is evident then, that in CPO-3, five interspecific crosses from CT occurred (5 GOT 2's), and the remainder of the progeny exhibiting PGI 2

(10 - 5 = 5) are the result of outcrossing from CPML. It should be noted that the occurrence of the parental phenotype in progeny is usually the result of selfing or a cross with a plant exhibiting the same phenotype within that taxon. CPO-1 has evidently crossed with all other station 14 plants, though, as seen by the presence of its GOT 4 phenotype. Segregating into type 3 and 5, GOT 4 produces type 3's and 4's in a 1:1 ratio when crossed with a GOT 3 homozygous plant.

Within the CPML progeny, interspecific crosses with CT are determined by the presence of a GOT 2 phenotype as found in CPML 3,4,7,11 and 12. The accurate verification of outcrossing from CPO into CPML is limited to one plant within station 14, that plant being CPO-1. The presence of PGI 2, GOT 4 and PER 4 in CPML 9 and 12 indicate the occurrence of gene flow from CPO-1 to CPML. The presence of plant CPML-2, however, with its type 2 PGI phenotype made it impossible to determine by isozyme analysis alone if the remaining type 2 PGI progeny within the CPML were the result of gene flow from CPO or from plant 2 within the taxon.

Analysis of progeny within the CT group revealed interspecific gene flow from station 5 CPML by the presence of PGI 1 phenotypes in association with GOT 2 phenotypes in CT-2 and 3. As for crossing with CPO, progeny from CT-7 and 15 offer firm proof of gene flow from station 14 because of their PGI 2 phenotype. CT-7 obviously had to cross with CPO-1 in order to exhibit the GOT 4 phenotype of that plant, while the GOT 2 of CT-15 is the result of a cross from a CPO having a GOT 3 phenotype.

The unique slow bands of PER marker phenotypes 4 and 5 in CPO provided firm identification of this taxon as the pollen parent in hybrid

progeny from fruits of both CPML and CT. However, CPO germplasm was not always detectable with the PER system because PER phenotype 4 is a heterozygous phenotype. In hybridization with plants carrying PER 3, progeny will shown PER 3 and PER 4 in a 1:1 ratio.

Table 4 summarizes the frequency of intertaxon genetic exchange events observed in the number of progeny sampled from each taxon. The identity of the pollen parent was determined as above, and the distance over which gene flow occurred was measured.

Table 1

Potential pollinators caught in association with experimental plants. All specimens vouchered in the Entomology Department Insect Collection, Texas A&M University.

Table 1

Collection # (H. Wilson)	Insect Species Identification	Sex of Bee	Associated Plant	Station at Which Caught	Date of Collection
3925	Melissodes b. bimaculata (Lepeletier)	F	texana	12	12 07 82
3926	Peponapis pruinosa (Say)	F	melopepo	05	09 07 82
3927	Peponapis pruinosa (Say)	M	melopepo	05	09 07 82
3928	Peponapis pruinosa (Say)	M	melopepo	05	30 06 82
3929	Xenoglossa strenua (Cresson)	F	melopepo	05	01 07 82
3930	Xenoglossa strenua (Cresson)	F	melopepo	16	09 07 82
3931	Xenoglossa strenua (Cresson)	F	melopepo	05	29 06 82
3932	Xenoglossa strenua (Cresson)	M	melopepo	05	29 06 82
3933	Xenoglossa strenua (Cresson)	F	melopepo	05	30 06 82
3934	Xenoglossa strenua (Cresson)	M	melopepo	05	01 07 82
3935	Xenoglossa strenua (Cresson)	F	ovifera	14	27 07 82
3936	Xenoglossa strenua (Cresson)	F	ovifera	14	31 07 82
3937	Xenoglossa strenua (Cresson)	M	texana	15	03 09 82
3938	Xenoglossa strenua (Cresson)	F	texana	03	17 07 82
3939	Xenoglossa strenua (Cresson)	F	melopepo	05	03 09 82
3940	Xenoglossa strenua (Cresson)	F	texana	01	29 07 82
3941	Peponapis pruinosa (Say)	F	ovifera	14	17 07 82
3942	Bombus p. pennsylvanicus (Degeer)	?	melopepo	16	17 07 82
3943	"skipper"	?	ovifera	14	27 07 82
3944	Xenoglossa strenua (Cresson)	F	ovifera	14	22 07 82
3946	Apis mellifera Linnaeus	?	texana	03	25 07 82
3948	Agapostemon texanus (Cresson)	?	texana	02	15 07 82
3949	Agapostemon texanus (Cresson)	?	melopepo	05	24 07 82
3950	Hemithalictus lustrans (Cockerell)	?	texana	10	23 07 82
3951	Hemithalictus lustrans (Cockerell)	?	ovifera	14	28 07 82

Table 2

Data showing how bee movement was monitored within and between stations of experimental plants using various color markers.

Table 2

STATION	TAXON	MARKER COLOR	#BEES* MARKED	#TIMES MARKED BEES RE-CAUGHT**	#TIMES BEE WITH DIFFERENT MARKING CAUGHT	#TIMES BEE WITH DIFFERENT MARKING SIGHTED	#SIGHTINGS OF OTHERS APIS	BOMBUS	"SKIP"	AGAPOSTEMON	HEMITHALICTUS
1-4	CT	pink	08	03	02(white)	01	0	0	0	0	0
5	CPML	white	18	11	00	02(pink)	0	0	0	1	0
6	CT	none	00	00	00	00	0	0	1	0	0
7	CT	none	00	00	00	01(blue)	0	0	2	0	4
9	CT	none	00	00	00	00	0	0	0	0	3
10	CT	orange	01	01	00	00	0	0	0	0	4
12	CT	none	00	00	00	00	0	1	0	0	0
14	CPO	blue	17	27	03(white)	00	2	3	4	0	2
15	CT	blue	04	01	00	00	0	0	0	0	0

*Xenoglossa

**does not refer to #of bees, rather number of instances of re-catching

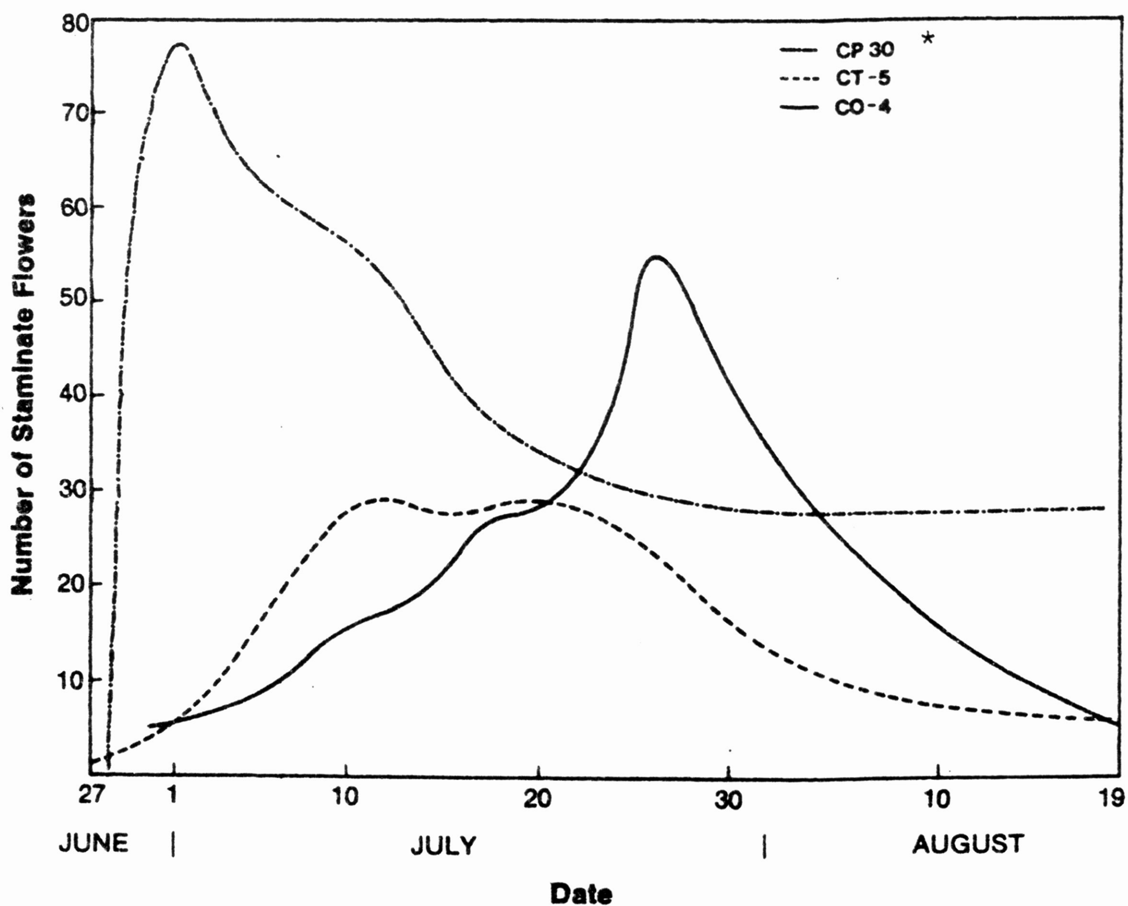


Fig 3

Staminate flowering phenology allowing comparison of the flowering activity of the three** taxa throughout the growing season.

*CP30=CPML
CT-5=CT
CO-4=CPO

**CPMD was eventually omitted from the study as it became evident that plants of this taxon did not become well established.

Table 3

Illustrates the results obtained upon electrophoresis of progeny from experimental plants. The number of progeny sampled from each parent plant is shown according to electrophoretic phenotype expressed for each enzyme system, e.g., 48 progeny from plant CPML-6 were sampled, and all expressed the phenotype of the parent plant in PGI, GOT, and PER systems. These phenotypes being indicative of the taxon, no intervarietal or interspecific crossing appears to have occured.

+ Denotes heterozygous phenotype within that enzyme system

* Denotes the phenotype of the parent plant

Table 3

Electrophoretic Results By Plant & Taxon

Parent	PGI			GOT					PER				
Plant	1	2+	3	1	2+	3	4+	5	1	2	3+	4+	5
CPO-1			36*			9	12*	5				7	29*
CPO-2			47*			12*	1					10	32*
CPO-3		10	141*		5	122*	23				35	73*	36
CPO-4		3	39*		3	25*	14					11	29*
CPO-5		23	123*		9	83*	52				21	86*	32
CPO-TOTAL		36	386		17	251	102	5			56	187	158
CPML-1	26*	11				37*					37*		
CPML-2	16	26*	2			44*					44*		
CPML-3	74*	1			1	74*					75*		
CPML-4	49*	1			1	49*					50*		
CPML-5	26*					16*					26*		
CPML-6	48*					48*					48*		
CPML-7	63*				1	62*					63*		
CPML-8	21*					21*					10*		
CPML-9	28*	2				18*	2				28*	2	
CPML-10	12*					12*					12*		
CPML-11	71*	1			4	68*					59*		
CPML-12	23*	3			11	14*	1				25*	1	
CPML-13	19*					9*					19*		
CPML-14	12*					-*					12*		
CPML-15	37*					22*					37*		
CPML-16	3					3					-		
CPML-TOTAL	528	45	2		18	507	3				545	3	

Electrophoretic Results-cont.-

Parent Plant	PGI 1	PGI 2+	PGI 3	GOT 1	GOT 2+	GOT 3	GOT 4+	GOT 5	PER 1	PER 2	PER 3+	PER 4+	PER 5
CT-1	56*			56*						55*	1		
CT-2	38*			28*	10				28*		10		
CT-3	100*			99*	1				6	22	72*		
CT-4	62*			48*					12	12	38*		
CT-6	30*			30*						30*			
CT-7	19*	14		19*			14			4*	15	14	
CT-9	82*			72*					14	14	54*		
CT-15	71*	1		59*	1				6	9	56*	1	
CT-TOTAL	458	15		411	12		14		66	146	246	15	

Table 4

Observed frequency of gene flow between taxa with source of foreign pollen and distance from pollen source to egg parent.

* Determination of specific pollen source within taxon not possible with available data.

Table 4

Taxon	Number of Progeny Sampled	% Showing Genetic Exchange	Pollen Source	Distance of Gene Flow (m)
CPO	422	4.30	CT	>15
		4.50	CPML	1410 or 1315*
CPML	575	3.13	CT	>100
		.52	CPO	1410 or 1160*
CT	473	2.33	CPML	<200
		3.17	CPO	565

IV. DISCUSSION

Xenoglossa and Peponapis, commonly known as the "squash" or "gourd bees", are two genera of solitary bees that have evidently co-evolved with the Cucurbita (Hurd et al. 1971) and preferentially, if not totally, subsist on its nectar and pollen. It was indeed interesting to find that one of these genera, Xenoglossa, held such an irrefutable place as primary pollinator over the others in this study area. Shear supremacy in numbers of Xenoglossa over Peponapis, and the fact that honeybees have a difficult time removing pollen from the anthers of Cucurbita (Linsley 1960), resulting in much smaller pollen loads in comparison to Xenoglossa, makes for a genuine lack of competitors for this position. It was with this in mind that Xenoglossa was selected to monitor "pollinator" movements within and between the stations.

The high frequency with which marked bees were re-caught at the larger stations, 5 and 14, and the fact that these two stations continually exhibited more pollinator activity in comparison with the smaller stations is consistent with the proposed correlation between resource quality and pollinator flight movements (Pyke 1978). Because Cucurbita is distinctive in that its flowers open early in the morning and quickly wilt as temperatures rise and light intensity increases (Hurd et al. 1971), Xenoglossa could be consistently found in high numbers early in the morning at the larger sites where their reward in nectar and pollen was greatest. Conversely, at smaller stations, where flowers were produced sporadically or only in small numbers, pollinator activity also was observed to be low and sporadic.

It still remains, however, that pollinator movement between stations

was observed. This can most likely be explained by the fact that no station established in this study could accurately be described as dense enough to keep the high number of bees in one area. The marked bees probably visited the highest resource quality areas first, and then had to "hustle" for their food, foraging over the entire area. The low plant density situation in the CT stations and their relatively distant spacing from each other combines to reduce the probability of intrataxon crossing and increases the likelihood of pollinator movement toward a higher resource area, stations 5 and 14. This hypothesis is consistent with observation of bee movement and electrophoretic evidence of gene flow between stations.

The flowering phenology, when combined with the isozyme data (Appx. IV), revealed that pistillate flowers fertilized at a time when the number of staminate flowers within all taxa was low had the highest probability of undergoing genetic exchange. This is almost surely the result of long distance pollen carryover between taxa by bees as a result of low resource quality.

One note of interest is the rather significant event of what appears to be crosses from CPO into CPML at a time before June 28. Staminate flower production within the CPO group was not observed before June 28. This may be accounted for by station 16, a pre-existing establishment of zucchini like Cucurbits discovered later in the summer. For the purposes of this study they have been incorporated into both the CPO and CPML groups due to the fact that the station was destroyed before it could be genetically assessed.

In considering the overall meaning of the electrophoretic results, it

should be noted that interspecific gene flow can and does occur between the *C. pepo* complex and *C. texana* under natural conditions involving natural pollen vectors. The distances over which both interspecific and intervarietal crossing occurred were great enough to effectively dissolve the boundaries of the two populations of *Cucurbita* established and thus allow consideration of possible genetic exchange between all stations. The fact that gene flow was observed to occur at distances well over the published isolation requirements for *Cucurbita* may possibly be due to the fact that the isolation distance was established for the genus as a crop. The current belief is that gene flow between natural populations may be higher than in crops over the same distance (Levin 1981). The results of this study certainly confirm this belief. The effects of such gene flow between populations, even if the levels were low from each source, are additive, and in the end may have a considerable impact on the genetic structure of the recipient population (Levin, 1981).

V. CONCLUSIONS

When the biological criteria are used to delimit a species, one must have a means of directly measuring the parameters of genetic exchange between groups of organisms. In the past, this has proved to be an area of great tribulation among taxonomists and evolutionary biologists alike, who tended to rely most heavily on gross morphological characteristics to define a species. In areas such as the paleo-sciences, a method that relies on accurate determination of genetic exchange is hardly likely. However, for those interested in more contemporary puzzles, a practical solution to the problem of measuring gene flow among and between plant populations would be through isozyme analysis. The technique of gel electrophoresis is certainly one of the fastest and most accurate means of quantifying this event, and undoubtedly has many applications in the fields of taxonomy and evolutionary systematics.

The use of isozyme analysis has shown that gene flow between *C. texana* and the *C. pepo* complex can and does occur under both agricultural and natural conditions, and raises serious questions as to their present classification as separate and distinct species.

Furthermore, and in conclusion, the published estimates of distance limits to *Cucurbita* gene flow are incorrect, and any subsequent modification should include isolation requirements necessary for the genus under natural conditions.

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APPENDIX I.

Buffer Solution Preparation

PK-G (Poulik Gel Buffer pH=8.7)

200 g. Tris (0.083m)

22 g. Citric Acid (0.005m)

19 L. H_2O

Fill for total volume of 20.0 L with H_2O

PK-E (Poulik Electrode buffer pH = 8.1)

482.3 g. H_3BO_3 0.3M (Granulor) Boric Acid

62.4 g. NaOH 0.056m Sodium Hydroxide

25.0 L. H_2O

Fill for total volume of 26.0 L. with H_2O

Stain Buffer (0.1 M Tris HCL pH = 8.0)

96.8 g Tris

7.0 L H_2O

ca. 45 ml HCL (conc)

pH to 8.0 with HCL L. Fill for total volume of 8.0 L. with H_2O

APPENDIX II.

Procedure Followed in Electrophoresis Using a Thin (3 Slice) Gel.

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1. Prepare the buffer solutions (gel, electrode, and stain)

2. Gel preparation

   a. Firmly seal gel mold electrode slots with masking tape and apply thin even film of glycerin on base plate using kim-wipes.
   b. In a 1L vacuum flask, add 39.5 g hydrolyzed potato starch with approximately 100 ml of 395 ml gel buffer and mix into a slurry.
   c. Heat over a bunsen burner the remaining gel buffer in a 1L volumetric flask until just prior to a rolling boil.
   d. Quickly, yet carefully, pour the heated buffer into the vacuum flask containing the slurry while continuously mixing.
   e. Immediately stopoper the vacuum flask, attach to a vacuum pump and aspirate while still mixing and occasionally heating until the small air bubbles are gone. Care must be taken not to cook the starch.
   f. Pour into the prepared gel mold, remove with fine forceps any foreign matter or cooked starch while still hot, and allow to cool 30-40 minutes.
   g. Cover gel with saran wrap and allow to further set for 2-48 hours before loading samples.

3. Sample preparation and gel loading

   a. Collect and label samples to be run.
   b. Homogenize samples and absorb onto wicks.
   c. Cut slit along the width of the gel at the origin and insert the wicks using fine forceps.

4. Gel running

   a. Fill electrode trays in refrigerated cabinet with chilled electrode buffer.
   b. Allow power supply to warm up.
   c. Remove masking tape seal.
   d. Place loaded gel in proper + / - orientation in the electrode trays, check circuitry, and set power pack at 55 milliamps and 250 volts for the duration of the run.
   e. Remove wicks after 30 minutes.

5. Gel slicing and staining

   a. Carefully remove gel from its mold and cut a notch in standardized corner for orientation.
   b. Horizontally slice on a plexiglass slicing bed.
   c. Place separate slices into staining trays
   d. Add pre-weighed stain to warmed stain buffer
   e. Let incubate at $38^{o}C$ and occasionally agitate until sites of activity reach optimum visibility.
   f. Decant stain, rinse slice, and fix in 30% ETOH.

6. Score the phenotypes and/or photograph gel.

APPENDIX III.

Stain Assay Procedures

1. Phosphoglucose Isomerase (PGI) - 1 gel

   a. 1ml PMS
   b. 1ml MTT
   c. 1ml Fructose-6-phosphate
   d. 1ml NADP
   e. 2 drops G-6-PDH
   f. 50ml Stain Buffer (warm)

After slicing, add PMS, MTT, and F-6-P to heated stain buffer. Just prior to staining, add NADP and G-6-PDH to stain buffer. Pour over gel and incubate for 30 minutes. Rinse and fix in 40% EtOH.

2. Glutamate-Oxaloacetate Transaminase (GOT) - 2 gels

   a. GOT Substrate Mixture
   160mg L-Aspartic Acid
   600mg PVP
   60mg EDTA
   1.7g Na_2HPO_4

   b. Substrate Solution
   60ml H_2O (warm)
   45mg alpha-Ketoglutaric Acid
   1 flask Substrate Mixture
   100mg Fast Blue BB Salt (GOT Capsule)

After slicing, add a-Ketoglutarate and GOT capsule to flask of substrate mixture. Add the warm water, mix, and pour over two gels, dividing the stain evenly between them. Incubate for 15 minutes. Rinse and fix in 50% glycerol.

3. Peroxidase (PER) - 1 gel

   a. Benzidine Stock Solution
   2mg Benzidine Dihydrochloride*
   18ml Glacial Acetic Acid
   (Let dissolve 1 hour before adding H_2O)
   72ml H_2O

   b. Substrate Solution
   30ml H_2O (warm)
   10ml 0.6% Hydrogen Peroxide
   4ml Benzidine Stock Solution
   2.5mg Ammonium Chloride

Mix substrate solution and pour over gel. Stain 10 minutes, decant stain into biohazard container, fix in 30% EtOH.

*Benzidine is a possible carcinogen.

APPENDIX IV.

Electrophoretic Results Arranged According To Seed Packet (Fruit) And Date Of Electrophoresis

ISOCU-J

1. 1=TAXON/STRAIN IDENTIFICATION (CO=CPO, CP=CPML, CT=CT)
2. 2=INDIVIDUAL PLANT IDENTIFICATION NUMBER
3. 3=DATE OF PISTILLATE FLOWERING
4. 4=FRUIT (SEED PACKET) IDENTIFICATION
5. 5=DATE OF ELECTROPHORESIS

					PGI			GOT					PER					
8.	11111	2222	3333	44	1	2	3	1	2	3	4	5	1	2	3	4	5	555555
10.	CO 04	0013	1907	AJ	00!	00!	10!	00!	00!	00!	00!	00!	00!	00!	00!	01!	09!	141082
11.	CO 04	0013	2407	AK	00!	00!	10!	00!	00!	03!	07!	00!	00!	00!	00!	00!	10!	161282
12.	CO 04	0013	2407	AK	00!	00!	04!	00!	00!	00!	00!	04?	00!	00!	00!	01!	03!	281082
13.	CO 04	0013	2507	AL	00!	00!	12!	00!	00!	06!	05?	01!	00!	00!	00!	05!	07!	121082
14.	CO 04	0206	1407?	LL	00!	00!	11!	00!	00!	00!	00!	00!	00!	00!	00!	02!	09!	081082
15.	CO 04	0206	1607	MM	00!	00!	10!	00!	00!	00!	00!	00!	00!	00!	00!	00!	10!	081082
16.	CO 04	0206	1607	J	00!	00!	05!	00!	00!	05!	00!	00!	00!	00!	00!	00!	00!	160982
17.	CO 04	0206	2607	OO	00!	00!	12!	00!	00!	00!	00!	00!	00!	00!	00!	06!	06!	081082
18.	CO 04	0206	2807	PP	00!	00!	01!	00!	00!	00!	00!	00!	00!	00!	00!	01!	00!	081082
19.	CO 04	0206	2807	PP	00!	00!	08!	00!	00!	07!	01?	00!	00!	00!	00!	01!	07!	121082
20.	CO 04	0208	0407	RR	00!	01!	14!	00!	00!	06!	09?	00!	00!	00!	02!	08!	05!	121082
21.	CO 04	0208	1007	SS	00!	00!	15!	00!	00!	14!	01?	00!	00!	00!	05!	06!	04!	121082
22.	CO 04	0208	1007	TT	00!	00!	14!	00!	00!	14!	00!	00!	00!	00!	02!	04!	04!	281082
23.	CO 04	0208	1007	TT	00!	00!	12!	00!	00!	09!	03!	00!	00!	00!	01!	05!	06!	121082
24.	CO 04	0208	1007	SS	00!	00!	07!	00!	00!	05!	01?	00!	00!	00!	02!	03!	02!	021182
25.	CO 04	0208	1107	UU	00!	00!	06!	00!	00!	06!	00!	00!	00!	00!	03!	02!	01?	281082
26.	CO 04	0208	1107	UU	00!	01!	01!	00!	01!	01!	00!	00!	00!	00!	02!	00!	00!	121082
27.	CO 04	0208	1207	VV	00!	01!	12!	00!	01!	12!	00!	00!	00!	00!	03!	04!	03!	281082
28.	CO 04	0208	1207	VV	00!	00!	13!	00!	00!	13!	00!	00!	00!	00!	03?	07!	03!	121082
29.	CO 04	0208	1307	WW	00!	01!	10?	00!	00!	09!	02?	00!	00!	00!	02!	08!	01!	121082
30.	CO 04	0208	1307	WW	00!	01!	14!	00!	00!	11!	04!	00!	00!	00!	01!	11!	03!	021182
31.	CO 04	0208	1307	XX	00!	02!	13!	00!	01!	12!	02!	00!	00!	00!	05!	06!	04!	021182
32.	CO 04	0208	1307	XX	00!	03!	10!	00!	02!	10!	01!	00!	00!	00!	04!	09!	00!	121082
33.	CO 04	0309	1007	AB	00!	00!	05!	00!	00!	03?	02?	00!	00!	00!	00!	01!	04!	121082
34.	CO 04	0309	1007	AB	00!	00!	14!	00!	00!	07!	07!	00!	00!	00!	00!	04!	08!	281082
35.	CO 04	0309	1607	AC	00!	00!	04!	00!	00!	04!	00!	00!	00!	00!	00!	00!	04!	121082
36.	CO 04	0309	1607	AC	00!	00!	08!	00!	00!	08!	00!	00!	00!	00!	00!	00!	08!	141082
37.	CO 04	0309	2207	BY	00!	03!	08!	00!	03!	03!	05!	00!	00!	00!	00!	06!	05!	281082
38.	CO 04	0311	0207	YY	00!	00!	01!	00!	00!	00!	00!	00!	00!	00!	01!	00!	00!	281082
39.	CO 04	0311	0207	YY	00!	00!	07!	00!	00!	07?	00!	00!	00!	00!	00!	04!	03!	121082
40.	CO 04	0311	0407	BU	00!	00!	03!	00!	00!	00!	03!	00!	00!	00!	00!	02!	01!	140283
41.	CO 04	0311	1007	BW	00!	00!	13!	00!	00!	05!	08!	00!	00!	00!	03!	08!	02!	261082
42.	CO 04	0311	1107	AD	00!	00!	12!	00!	00!	03!	09!	00!	00!	00!	01!	06!	05!	141082
43.	CO 04	0311	1107	AD	00!	00!	10!	00!	00!	03!	07!	00!	00!	00!	00!	07!	03!	021182
44.	CO 04	0311	1207	AE	00!	00!	13!	00!	00!	03!	10!	00!	00!	00!	02!	05!	05!	281082
45.	CO 04	0311	1207	AE	00!	00!	10!	00!	01?	01!	08!	00!	00!	00!	00!	07!	03!	141082
46.	CO 04	0311	1307	AF	00!	04!	09!	00!	00!	11!	02!	00!	00!	00!	02!	06!	02!	281082
47.	CO 04	0311	1307	AF	00!	06!	08!	00!	01!	11!	02?	00!	00!	00!	04!	08!	02!	141082
48.	CO 04	0311	1407	AH	00!	03!	06!	00!	01?	07!	01!	00!	00!	00!	00!	09!	01!	161282
49.	CO 04	0311	1407	AH			01?											161282
50.	CO 04	0311	1407	AH	00!	01!	09!	00!	01!	08!	01!	00!	00!	00!	00!	09!	01!	171282
51.	CO 04	0311	1407	ZZ	00!	00!	02!	00!	00!	02!	00!	00!	00!	00!	00!	02!	00!	121082
52.	CO 04	0311	1407	ZZ	00!	01!	03!	00!	01?	03!	00!	00!	00!	00!	03!	00!	01!	281082
53.	CO 04	0311	1407	ZZ	00!	05!	09!	00!	02!	12!	00!	00!	00!	00!	02!	08!	01!	281082
54.	CO 04	0311	1407	ZZ	00!	03!	07!	00!	02!	06!	01!	00!	00!	00!	03!	05!	02!	171282
55.	CO 04	0311	1407	ZZ						01?								171282
56.	CP		0016	L	03!	00!	00!	00!	00!	03!	00!	00!	00!	00!	00!	00!	00!	160982
57.	CP 30	0109	<2806	M	08!	02!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
58.	CP 30	0109	<2806	M	05!	04!	00!	00!	00!	09!	00!	00!	00!	00!	09!	00!	00!	300982

59.	CP 30 0109<2806	N	09!	01!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
60.	CP 30 0109<2806	N	04!	04!	00!	00!	00!	08!	00!	00!	00!	00!	08!	00!	00!	300982
61.	CP 30 0110 2906	P	04!	03!	00!	00!	00!	07!	00!	00!	00!	00!	07!	00!	00!	300982
62.	CP 30 0110 2906	P	05!	04!	01!	00!	00!	09!	00!	00!	00!	00!	09!	00!	00!	280982
63.	CP 30 0110 2906	BQ	02!	13!	00!	00!	00!	15!	00!	00!	00!	00!	15!	00!	00!	261082
64.	CP 30 0110 0908	CD	05!	04!	01!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	140283
65.	CP 30 0110 0908	CD	00!	02!	00!	00!	00!	03!	00!	00!	00!	00!	03!	00!	00!	110283
66.	CP 30 0111 0307	BO	08!	00!	00!	00!	00!	08!	00!	00!	00!	00!	08!	00!	00!	261082
67.	CP 30 0111 0307	BO	13!	01!	00!	00!	00!	12!	00!	00!	00!	00!	14!	00!	00!	260183
68.	CP 30 0111 0307	BO						02?								260183
69.	CP 30 0111 0607	Q	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	300982
70.	CP 30 0111 0607	Q	16!	00!	00!	00!	00!	16!	00!	00!	00!	00!	16!	00!	00!	261082
71.	CP 30 0111 0607	Q	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
72.	CP 30 0111 ??09	CE	17!	00!	00!	00!	01!	16!	00!	00!	00!	00!	17!	00!	00!	140283
73.	CP 30 0112 2806?	R	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
74.	CP 30 0112 2806?	R	14!	00!	00!	00!	00!	14!	00!	00!	00!	00!	14!	00!	00!	261082
75.	CP 30 0112 2806	R	09!	00!	00!	00!	01!	08!	00!	00!	00!	00!	09!	00!	00!	300982
76.	CP 30 0112 0107	S	06!	01!	00!	00!	00!	07!	00!	00!	00!	00!	07!	00!	00!	300982
77.	CP 30 0112 0107	S	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
78.	CP 30 0122 2806?	T	10!	00!	00!	00!	00!	00!	00!	00!	00!	00!	10!	00!	00!	280982
79.	CP 30 0122 0508	CA	16!	00!	00!	00!	00!	16!	00!	00!	00!	00!	16!	00!	00!	140283
80.	CP 30 0124 0607	U	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	140283
81.	CP 30 0124 0607	U	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
82.	CP 30 0124 0907	V	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
83.	CP 30 0124 0907	V	17!	00!	00!	00!	00!	18!	00!	00!	00!	00!	18!	00!	00!	110283
84.	CP 30 0124 0907	V	01?													110283
85.	CP 30 0201 2906	W	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
86.	CP 30 0201 0907	X	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	280982
87.	CP 30 0201 0907	Y	02!	00!	00!	00!	00!	02!	00!	00!	00!	00!	02!	00!	00!	280982
88.	CP 30 0201 0907	Y	08!	00!	00!	00!	00!	08!	00!	00!	00!	00!	08!	00!	00!	300982
89.	CP 30 0201 0907	Y	17!	00!	00!	00!	00!	17!	00!	00!	00!	00!	17!	00!	00!	110283
90.	CP 30 0202<2806	G	11!	00!	00!	00!	00!	11!	00!	00!	00!	00!	00!	00!	00!	160982
91.	CP 30 0202<2806	Z	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	300982
92.	CP 30 0203<2806	BP	14!	01!	00!	00!	00!	14!	01!	00!	00!	00!	14!	01!	00!	261082
93.	CP 30 0203<2806	BP	14!	01!	00!	00!	00!	14!	01!	00!	00!	00!	14!	01!	00!	040383
94.	CP 30 0207 0907	X	16!	00!	00!	00!	01?	15!	00!	00!	00!	00!	16!	00!	00!	110283
95.	CP 30 0305<2806	BB	12!	00!	00!	00!	00!	12!	00!	00!	00!	00!	12!	00!	00!	300982
96.	CP 30 0306<2806	E	13!	00!	00!	00!	00!	13?	00!	00!	00!	00!	00!	00!	00!	160982
97.	CP 30 0306 1607	CC	18!	00!	00!	00!	01?	17!	00!	00!	00!	00!	18!	00!	00!	110283
98.	CP 30 0306 1607	CC	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	300982
99.	CP 30 0306 1807	DD	20!	01!	00!	00!	03!	15!	00!	00!	00!	00!	21!	00!	00!	110283
100.	CP 30 0306 1807	DD						03?								110283
101.	CP 30 0306 1807	DD	10!	00!	00!	00!	00!	10!	00!	00!	00!	00!	10!	00!	00!	300982
102.	CP 30 0307<2806	EE	07!	01!	00!	00!	06!	02!	00!	00!	00!	00!	08!	00!	00!	300982
103.	CP 30 0307<2806	EE	16!	02!	00!	00!	05!	12!	01!	00!	00!	00!	17!	01!	00!	110283
104.	CP 30 0308 2906	FF	02!	00!	00!	00!	00!	00!	00!	00!	00!	00!	02!	00!	00!	081082
105.	CP 30 0308 0207	GG	08!	00!	00!	00!	00!	00!	00!	00!	00!	00!	08!	00!	00!	081082
106.	CP 30 0308 0207	GG	09!	00!	00!	00!	00!	09!	00!	00!	00!	00!	09!	00!	00!	140283
107.	CP 30 0313 2806	HH	12!	00!	00!	00!	00!	00!	00!	00!	00!	00!	12!	00!	00!	081082
108.	CP 30 0314 2906	JJ	08!	00!	00!	00!	00!	00!	00!	00!	00!	00!	08!	00!	00!	081082
109.	CP 30 0314 2906	JJ	05!	00!	00!	00!	00!	00!	00!	00!	00!	00!	05!	00!	00!	081082
110.	CP 30 0314 2707	KK	09!	00!	00!	00!	00!	09!	00!	00!	00!	00!	09!	00!	00!	110283
111.	CP 30 0314 2707	KK	02!	00!	00!	00!	00!	00!	00!	00!	00!	00!	02!	00!	00!	081082
112.	CP 30 0314 2707	KK	13!	00!	00!	00!	00!	11!	00!	00!	00!	00!	13!	00!	00!	140283
113.	CP 30 0314 2707	KK						02?								140283
114.	CT 05 0004 1707	BG	13!	00!	00!	12!	00!	00!	00!	00!	04!	05!	04!	00!	00!	310183
115.	CT 05 0007 2107	CF	18!	00!	00!	18!	00!	00!	00!	00!	00!	04!	14!	00!	00!	040383
116.	CT 05 0001 2107	BC	17!	00!	00!	17!	00!	00!	00!	00!	00!	16!	01!	00!	00!	040383
117.	CT 05 0001 2107	BE	13!	00!	00!	13!	00!	00!	00!	00!	00!	13!	00!	00!	00!	310183
118.	CT 05 0001 2107	BE	14!	00!	00!	14!	00!	00!	00!	00!	00!	14!	00!	00!	00!	090383
119.	CT 05 0001 2107	BC	12!	00!	00!	12!	00!	00!	00!	00!	00!	12!	00!	00!	00!	171282

120.	CT 05 0002	1007	AW	06!	00!	00!	06!	00!	00!	00!	00!	06!	00!	00!	00!	00!	161282
121.	CT 05 0002	1007	AW	03!	00!	00!	03!	00!	00!	00!	00!	03!	00!	00!	00!	00!	171282
122.	CT 05 0002	1507	BJ	14!	00!	00!	03!	10!	00!	00!	00!	04!	00!	10!	00!	00!	260183
123.	CT 05 0002	1507	BJ				01?										260183
124.	CT 05 0002	2007	AR	11!	00!	00!	11!	00!	00!	00!	00!	11!	00!	00!	00!	00!	161282
125.	CT 05 0002	2007	AR	04!	00!	00!	04!	00!	00!	00!	00!	04!	00!	00!	00!	00!	171282
126.	CT 05 0003	1307	BH	15!	00!	00!	15!	00!	00!	00!	00!	05!	04!	05!	00!	00!	260183
127.	CT 05 0003	1307	BH											01?			260183
128.	CT 05 0003	1407	BL	03!	00!	00!	03!	00!	00!	00!	00!	00!	00!	03!	00!	00!	310183
129.	CT 05 0003	1407	BL	11!	00!	00!	11!	00!	00!	00!	00!	02!	03!	06!	00!	00!	310183
130.	CT 05 0003	1407	BL	11!	00!	00!	11!	00!	00!	00!	00!	00!	03!	08!	00!	00!	040383
131.	CT 05 0003	1407	BL	06!	00!	00!	06!	00!	00!	00!	00!	00!	01!	05!	00!	00!	090383
132.	CT 05 0003	1607	AQ	14!	00!	00!	13!	01?	00!	00!	00!	00!	02!	12?	00!	00!	301182
133.	CT 05 0003	1607	AQ	15!	00!	00!	15!	00!	00!	00!	00!	00!	05!	10!	00!	00!	090383
134.	CT 05 0003	1707	BI	10!	00!	00!	10!	00!	00!	00!	00!	00!	02!	08!	00!	00!	310183
135.	CT 05 0003	1707	BI	02!	00!	00!	02!	00!	00!	00!	00!	00!	01!	01!	00!	00!	310183
136.	CT 05 0003	1707	BI	02!	00!	00!	02!	00!	00!	00!	00!	02!	00!	00!	00!	00!	260183
137.	CT 05 0003	1807	AU	10!	00!	00!	10!	00!	00!	00!	00!	02!	00!	05!	00!	00!	161282
138.	CT 05 0003	1807	AU	03!	00!	00!	03!	00!	00!	00!	00!	00!	01!	02!	00!	00!	171282
139.	CT 05 0003	1807	AU											03?			161282
140.	CT 05 0003	2907	AP	13!	00!	00!	13!	00!	00!	00!	00!	00!	04!	09?	00!	00!	301182
141.	CT 05 0004		BM	12!	00!	00!	12!	00!	00!	00!	00!	04!	03!	05!	00!	00!	310183
142.	CT 05 0004	1507	AX	04!	00!	00!	01!	00!	00!	00!	00!	04!	00!	00!	00!	00!	281082
143.	CT 05 0004	1707	BG				01?										310183
144.	CT 05 0004	1807	AV	12!	00!	00!	12!	00!	00!	00!	00!	00!	00!	12!	00!	00!	161282
145.	CT 05 0004	1807	AO	08!	00!	00!	00!	00!	00!	00!	00!	00!	00!	08!	00!	00!	111182
146.	CT 05 0004	1807	AO	13!	00!	00!	10!	00!	00!	00!	00!	00!	04!	09!	00!	00!	171282
147.	CT 05 0006	1307	BK	15!	00!	00!	15!	00!	00!	00!	00!	00!	15!	00!	00!	00!	260183
148.	CT 05 0006	1307	BK	15!	00!	00!	15!	00!	00!	00!	00!	00!	15!	00!	00!	00!	040383
149.	CT 05 0007	1509	CG	01!	14!	00!	01!	00!	00!	14!	00!	00!	00!	01!	14!	00!	040383
150.	CT 05 0009	0607	AM	04!	00!	00!	04!	00!	00!	00!	00!	04!	00!	00!	00!	00!	171282
151.	CT 05 0009	0607	AM									01?					161282
152.	CT 05 0009	0607	AM	10!	00!	00!	10!	00!	00!	00!	00!	03!	00!	06!	00!	00!	161282
153.	CT 05 0009	0607	AM	10!	00!	00!	00!	00!	00!	00!	00!	00!	00!	10!	00!	00!	111182
154.	CT 05 0009	1007	AY	11!	00!	00!	11!	00!	00!	00!	00!	02!	00!	07!	00!	00!	161282
155.	CT 05 0009	1007	AY	03!	00!	00!	03!	00!	00!	00!	00!	00!	00!	03!	00!	00!	171282
156.	CT 05 0009	1007	AY									01?		01?			161282
157.	CT 05 0009	1107	BN	03!	00!	00!	03!	00!	00!	00!	00!	00!	03!	00!	00!	00!	310183
158.	CT 05 0009	1107	BN	12!	00!	00!	12!	00!	00!	00!	00!	01?	03!	08!	00!	00!	310183
159.	CT 05 0009	1607	BD	12!	00!	00!	12!	00!	00!	00!	00!	02!	03!	07!	00!	00!	310183
160.	CT 05 0009	1607	BD	17!	00!	00!	17!	00!	00!	00!	00!	00!	05!	12!	00!	00!	090383
161.	CT 05 0015	1607	BF	15!	01!	00!	15!	01!	00!	00!	00!	00!	04!	11!	01!	00!	040383
162.	CT 05 0015	1607	BF	16!	00!	00!	16!	00!	00!	00!	00!	06!	02!	08!	00!	00!	310183
163.	CT 05 0015	2607	AN	12!	00!	00!	00!	00!	00!	00!	00!	00!	00!	12!	00!	00!	111182
164.	CT 05 0015	2607	AN	02!	00!	00!	02!	00!	00!	00!	00!	00!	00!	02!	00!	00!	171282
165.	CT 05 0015	2607	AN	13!	00!	00!	13!	00!	00!	00!	00!	00!	03!	10!	00!	00!	040383
166.	CT 05 0015	1408	AT	13!	00!	00!	13!	00!	00!	00!	00!	00!	00!	13!	00!	00!	161282