PROTEIN STARCH-GEL ELECTROPHORESIS IN SCLERACTINIAN CORALS: A REPORT ON TECHNIQUES AND TROUBLESHOOTING

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INTRODUCTION

Protein electrophoresis in scleractinian corals has been used mostly in studies of population structure and clonal variation (Stoddart, 1983, 1984a,b; Hunter, 1985; Ayre and Willis, 1988), asexual reproduction (Stoddart, 1983; Willis and Ayre, 1985; Ayre and Reising, 1986) self- and cross- fertilization (Stoddart et al., 1988), and immunogenetics (Heyward and Stoddart, 1985). Until recently, the pioneering electrophoretic studies of Lamberts (1979) and Ohlhorst (1984) were the only ones having a taxonomic objective. Allozyme electrophoresis is now being widely used as an important tool for separating cryptic and sibling species of corals (e.g., Ayre et al., 1991; Knowlton et al., 1992; Miller, 1992; Weil, 1992a,b; Van Veghel and Bak, 1993; Weil and Knowlton, 1994; Garthwaite et al., 1994, Stobart and Benzie, 1994). Nevertheless, all published electrophoretic studies of corals lack detailed descriptions of the techniques (or modifications of these) used and generally cite classic references to generalized protocols developed for vertebrates (e.g., Selander et al., 1971; Nichols et al., 1973; Harris and Hopkinson, 1976).

Herein we summarize in detail the methodology used and discuss problems encountered in the electrophoretic studies of three common scleractinian genera, *Montastraea*, *Porites* and *Tubastraea* (Weil, 1992a,b; Knowlton et al., 1992; Weil and Knowlton, 1994; Weil and Brunetti, unpub. data). A few individuals of species from six other genera that are common in the Caribbean and eastern Pacific (*Agaricia*, *Acropora*, *Manicina*, *Meandrina*, *Pavona*, and *Leptoseris*) also were screened. It was not possible to test all possible combinations of enzyme systems, gel-buffer systems, allozyme stains and tissue homogenization procedures. Nevertheless, protocols used for collection, storage and homogenization of tissues, preparation and running of gels, and staining of the different allozymes, are described in detail. Few of these methods are new, but changes from the standard protocols are detailed so that future users can avoid undue difficulties. Customizing protocols can consume substantial effort, time and material resources that otherwise could be available for the main study. Information and comparisons with other electrophoretic studies involving corals are presented. The electrophoretic results obtained have contributed to the better understanding of the systematics and genetics of three important genera of scleractinian corals in the Caribbean and eastern Pacific. We hope this will also open the door for a more extensive use of these techniques in solving identification problems in cryptic and sibling species of scleractinian corals and other marine invertebrates. All laboratory work was conducted during 1989-1990 in the Molecular Users Laboratory of the Smithsonian Tropical Research Institute (STRI) in Panamá.

FIELD PROCEDURES

Collection of Samples

Small portions (100 cm^2) of healthy-looking, living tissue were carefully cut off from massive colonies at narrow, dead areas, with the aid of a chisel and a heavy hammer. Generally, it was not necessary to kill the whole colony (or leave an open scar in a large colony) except when small specimens were collected. Each sample was labeled with previously numbered tags (colored flagging tape tied to rubber bands), and placed back-to-back in 10-gal buckets underwater. Ten branches were broken off from each ramose colony, then bound together with a wide rubber band and a flagging label. [Samples of these sizes yield sufficient tissues and coral skeleton for several, independent assays of each individual colony (e.g., electrophoresis, nucleic acids, histology, morphometrics, growth bands, and isotopic-ratio analyses). Smaller sample sizes are recommended if only biochemical and corallite morphometry is going to be done]. All specimens were kept in sea water until processed.

Overall, samples from 30 species belonging to nine genera of scleractinian corals were collected from 8 localities in the Caribbean (CA) and five in the eastern Pacific (EP) (Figure 1; Table 1). Relevant colony information such as coloration, form, habitat, depth and position on the substrate were noted *in situ* before collection The total number of colonies collected per day depended on many factors, including abundance and depth range of the species, reef location, time available underwater, weather, and the time necessary to remove the tissue samples from the specimens. A good review of general guidelines for the collection, transport and storage of animal and plant tissues is given by Dessauer et al. (1990).

Tissue Collection and Storage

To minimize any detrimental influences of freezing on macromolecules, tissues were frozen quickly after removal and thawed rapidly just before initiating gel runs. Before starting to remove the tissues, all cryogenic vials were labeled and all instruments and necessary materials were organized and kept handy, thereby minimizing exposure and processing time.

Contamination is a major concern and needs to be avoided. Before processing, each coral sample was first rinsed and/or thoroughly agitated in a bucket with fresh sea water to wash off foreign particles (e.g., organic detritus) on the colony surface. Then 1-2 ml of living coral tissue and skeletal material were scraped off the coral surface of massive colonies with the aid of a hard, sharp, aluminum spatula mounted on a wooden handle. Care was taken not to scrape to low into the skeleton to avoid contamination by boring sponges and endolythic algae. In ramose species, the tips of the branches, where the tissue is thicker, were either cut with the spatula or broken and crushed with long, smooth-tipped pliers. In species with long, tubular corallites, like *Tubastraea*, the upper edge of the wall was cut away allowing much of the polypal tissue to be extracted with pliers or forceps from the lower center of the calice. Each tissue sample was placed into a previously labeled cryogenic vial (1-2 ml). Four to six drops of an indicator-extractant-grinding buffer (modified from Stoddart, 1983, Appendix A) were then added and thoroughly mixed with the scraped tissue using a fine spatula, to help stabilize the proteins (Dessauer et al., 1990). Vials were either quickly returned to crushed ice or placed immediately into liquid nitrogen or dry ice.

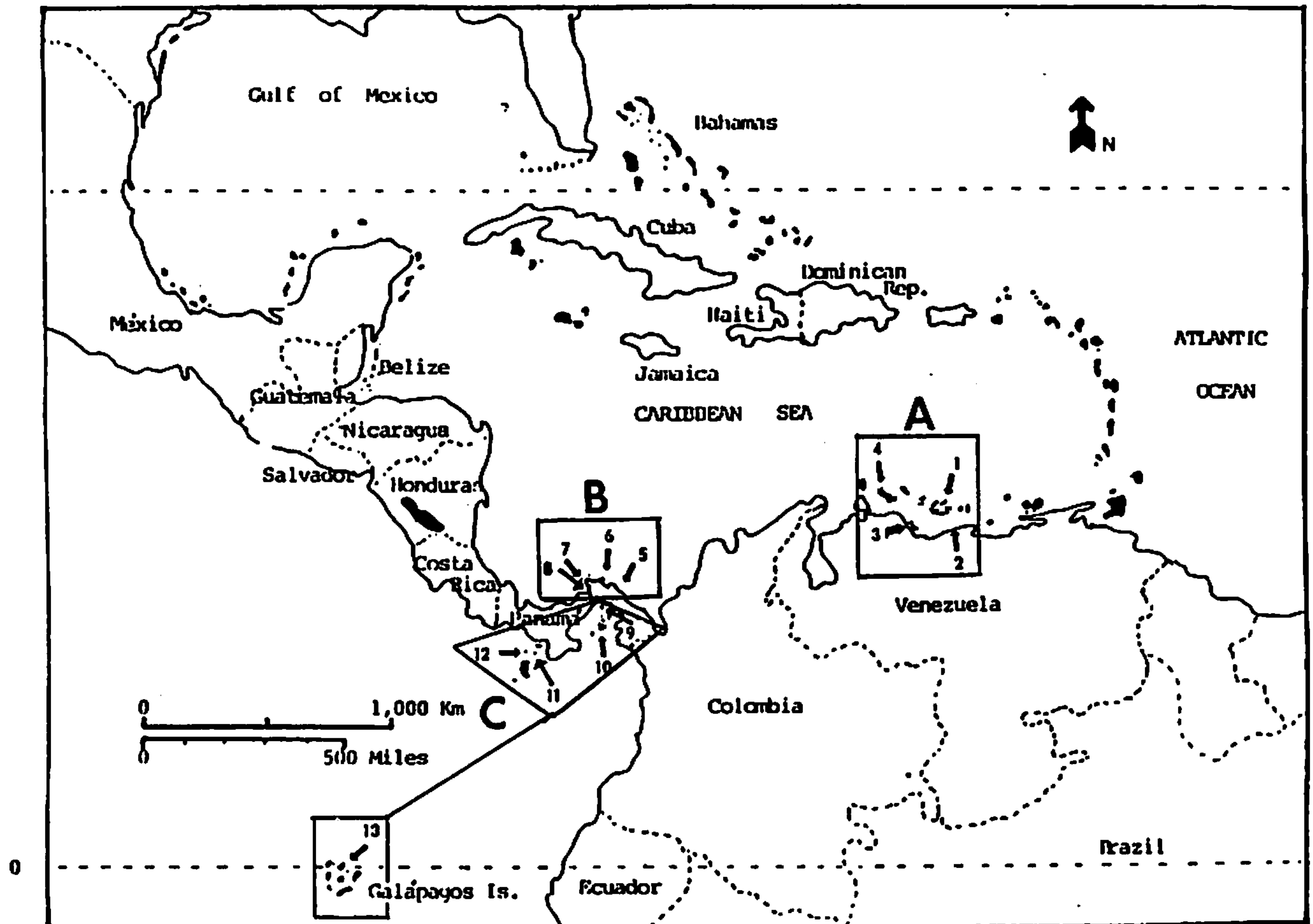

Figure 1. Geographic location of sampling localities in the Caribbean and eastern Pacific. Caribbean localities include the offshore Archipielago de los Roques National Park (1), and Tacoa (2), and the Morrocoy National Park (3) along the main coast of Venezuela; Curaçao (4) in the Netherland Antilles; Islas Salar (5), the Limones Keys and El Porvenir area (6) in the San Blas Archipelago in the east of the Caribbean coast of Panamá; the Portobello area (7) and the Galeta area (8) in the northern center of the Caribbean coast of Panamá. The eastern Pacific localities included the islands of Uraba (9) and Saboga (10) in the Gulf of Panamá, and the islands of Uva (11) and Secas (12) in the Gulf of Chiriqui on the Pacific side of Panamá; and the Galápagos Islands (13).

Table 1. Scleractinian coral species, locality and total number of colonies electrophoretically screened. CVe - Caribbean Venezuela, CCu - Caribbean Curaçao, CPa - Caribbean Panamá, PPa - Pacific Panamá, PGa - Pacific Galápagos.

Species	Locality	N
Acropora palmata	CPa	2
Acropora cervicornis	CPa	2
Agaricia agaricites	CPa	4
Agaricia humilis	CPa	4
Agaricia purpurea	CPa	4
Agaricia tenuifolia	CPa	2
Agaricia lamarcki	CPa	2
Agaricia grahamae	CPa	2
Agaricia undata	CPa	2
Leptoseris cucullata	CPa	4
Meandrina meandrites meandrites	CPa	3
Meandrina meandrites memorialis	CPa	3
Manicina aerolata	CPa	2
Montastraea annularis	CPa	59
Montastraea faveolata	CPa	61
Montastraea franksi	CPa	58
Montastraea cavernosa	CPa	34
Porites astreoides	CPa, CVe	176
Porites "branneri"	CVe, CCu	39
Porites colonensis	CPa	89
Porites porites	CPa, CVe	113
Porites furcata	CPa	108
Porites divaricata	CPa	51
Porites lobata	PPa, PGa	100
Porites panamensis *	PPa	30
Porites panamensis **	PPa	32
Pavona varians	PPa	4
Pavona sp.	PPa	4
Tubastraea coccinea	PPa	30
Tubastraea aurea	CVe	30
Tubastraea sp ***	PPa	20

*Population from Saboga island.

**Population from Uva island (See Weil, 1992a; 1992b).

***Allelic differences as well as significant differences in morphology and coloration were found in a population of small colonies of *Tubastraea* living in sympatry with *T. coccinea* in the eastern Pacific (Weil and Brunetti, unpub.data).

All instruments were rinsed (or agitated) in distilled water and thoroughly blotted with paper towels between samples.

Processing time for each specimen varied depending upon sample size, density of skeleton and instruments available. Between 20 and 30 colonies were processed in 2-3 man/hr during the same day the samples had been collected. At remote collection sites, vials with samples were kept on crushed ice until ten colonies had been processed, then all were dropped into the liquid nitrogen (to prevent excessive evaporation by frequent openings of the liquid nitrogen container lid). Also, when field time was limited, or dry ice or water ice were not available, small, labeled pieces (4 cm^2 in surface or a couple of branches) of each colony were put into plastic bags and dumped in the liquid nitrogen.

Samples were transported to the laboratory in liquid nitrogen containers, in dry-shippers or in styrofoam-protected coolers with dry ice. There, they were carefully organized in vial-holders or small boxes, properly labeled, and stored in the ultrafreezer at -80°C until the electrophoresis gel run.

LABORATORY PROTOCOLS

General information about the characteristics of the different enzymes and explicit, step-by-step instructions on how to establish a horizontal starch gel electrophoresis lab to perform protein electrophoresis, to stain for specific enzymes and non-enzyme proteins, and to interpret the resultant gels can be found in Harris and Hopkinson (1976), Ayala (1982), Richardson et al. (1986), and Murphy et al. (1990). Detailed explanations of experimental techniques and difficulties encountered during this study are provided below.

Gel Preparation

Gels were prepared the afternoon before the morning of the electrophoresis run. If samples were going to be run at night, gels were prepared the morning of the same day. After weighing and mixing the starch (SIGMA S-4501) and the buffer (15% w/v) (Appendices B and F) in a 1000 ml flask, the solution was thoroughly agitated until the starch was dissolved (this step can be done over an open flame). The starch-buffer solution was then cooked in a microwave oven set at high. Thirty second cooking intervals alternating with 10-15 s of strong agitation were used to improve starch dissolution and the homogeneity of the gel. Total cooking time depended on starch concentration, size of the gel, and power of the microwave oven (six minutes for a gel size of 500 ml and 15 % w/v in a 700 W microwave). When the gel was liquid and homogeneous, the flask was quickly moved to an aspiration hose and vacuumed for about 15-20 s while agitating vigorously to avoid aspirating gel out of the flask. The vacuum was then slowly released and the hot mixture rapidly poured into the center of a gel mold until it had filled and then, any obvious undissolved gel particles or large bubbles were quickly retrieved with a fine spatula. Gel molds were left on a flat, stable, horizontal surface to cool. Any gel with an excess of bubbles or undissolved gel particles was discarded and a new one prepared. While the gels were cooling down, electrode-buffer wells in electrophoretic trays were filled with appropriate buffer solutions and labeled. After the gels had solidified and cooled to room temperature, they were carefully

covered with plastic food wrap to prevent dehydration and bacterial contamination and stored at 4°C in an upright, sliding-door refrigerator.

Tissue Homogenization

Vials (or small pieces of corals), about ten at a time, were briefly placed on crushed ice at room temperature. Then a small portion of the still frozen tissue was removed from the vial with a fine spatula (or scrapped off the coral sample surface) and placed in a shallow well in a chilled ceramic grinding plate. One well was left between each sample to prevent contamination during the homogenization process. The order and orientation of the six samples on the plates was noted.

One to three volumes (of tissue volume) of Stoddart's (1983) cold extractant-grinding buffer were added to each sample. The tissue (including any carbonate skeleton) and buffer were then ground with glass pestles by hand until thoroughly homogenized (usually 2-4 min/sample). [At this stage, the coral mucus usually becomes a problem. It sticks to the glass rods and the walls of the plates, and if not enough grinding buffer has been added, the homogenate looks like a gel blob with a little free liquid solution. This condition varied among taxa with some species like *Porites panamensis* and *P. "branneri"* (Weil, 1992a, b) having significantly less mucus and therefore, producing more diluted homogenates. Ahermatypic species like *Tubastraea, Phylangia,* and *Astrangia*, have less mucus and softer exoskeletons, are easier to grind, and produce a dilute homogenate instead of a sticky glob (Bauchamp, pers. comm.)]. Tissues were homogenized and used immediately or returned to the ultrafreezer as soon as the plates were covered with plastic food wrap and labeled. The additional, short thawing period was not detrimental to our samples. Early the next morning, the grinding plates were taken out of the ultrafreezer one at a time, placed on a bed of crushed ice in an aluminum tray and left to thaw for a few minutes.

After all samples in one plate were homogenized, a 4 cm^2 square piece of Miracloth filter tissue was placed on top of each homogenate. Small pieces (size depended on gel size being used) of Whatman # 5 filter paper wicks were placed on top of the miracloth and pressed down until they were saturated with the homogenate. [Miracloth (Calbiochem Inc.), a nylon filter designed for dense substances, prevented high amounts of mucus from adhering to the wicks]. If many samples were being processed (e.g., 25 per day), the plate was kept at -20°C until the rest of the samples were processed.

Gel Loading and Running

A clean cut was made across the gel at about 5 cm from the cathodal side with a blunt scalpel and both gel pieces were pulled apart gently with the aid of paper towels. Wicks were blotted dry by placing them between paper towels and pressing. Then, the wicks were quickly and carefully loaded (in sequence from left to right, keeping the original order of the samples) into the gel using narrow-tip forceps. A marked ruler was used as a guide for loading the wicks at evenly spaced intervals (3-5 mm). Each gel contained a control, usually a sample from one individual that consistently showed good bands for all enzymes being assayed (and was, therefore, a good control for scoring the different alleles of the same locus across different specimens). In these assays, extracts of the ahermatype *Tubastraea coccinea* were used as the control [It has no zooxanthellae, mucus is scarce, the tissue/skeleton volume ratio is high, and it yielded good bands for almost all enzymes assayed. One small colony (25-30 cm^2 in surface area) renders enough

tissue to fill several 2 ml cryogenic vials. A very small volume (0.2 ml) of tissue extracted from the vial and grounded with two volumes of the grinding buffer is enough for one day of electrophoresis (6-8 gels)]. Other controls, usually one of the samples, were used together with *T. coccinea* throughout these experiments. A wick with tracking dye (0.1% bromophenol-blue) was placed at the edge of the gel to keep track of the migration. The bromophenol-blue can also be added to the grinding buffer.

After loading all samples, a plexiglas spacer (5 mm wide) was introduced between the gel and the anodal wall of the gel mold to press the wicks between the two pieces of gel at the loading area. Gels were partially covered with plastic wrap, leaving 1.5 in of uncovered gel for the sponges or buffer wicks, and then set in the corresponding electrophoresis apparatus in the sliding-door refrigerator. Each apparatus was connected to an independent power supply whose voltage and amperage were controlled. Common cleaning sponges were used to wick the buffer solution and establish contact with the gel. The system was then completely covered with a large piece of plastic wrap, and an aluminum tray filled with crushed ice was placed on top of the gel. After checking that everything was ready, the system was turned on. All systems were run at 4°C for 4-8 hr depending on the voltage or amperage and the gel buffers. Electric current was constant throughout the runs and at intensity settings that would not warm up the gels too much. Table 2 shows running times and electrical current settings used for gels of different sizes (ml) and concentrations (% w/v) at the STRI lab.

Table 2. Summary table for gel-buffer systems, pH, gel size (ml), gel concentration (%), current settings and enzymes with good and regular resolution.

GEL	TC[1]	LIOH[2]	TVB[3]	CT[4]	RW[5]
PH	8.0	8.4	8.0	6.0/6.1	8.5
SIZE	500	500	500	300	500
%	15	15	15	15	15
mA	80	--	50	50	--
mV	--	325	--	--	275
ENZYMES	GPI	LTY	ODPH	GPI	LTY
	PGM	LGG	G3PGH	MDH	LGG
	MDH	LPP	MDH	6PGH	LPP
	MDHP	TPI	MDHP	MDHP	TPI
	GDH	EST	GDH	ACP	EST
	6PGH	LVP	CAP	LDH	LVP
	SOD	SOD	ACP		MPI
	LDH	MPI	PGM		LLP

[1]Tris-citrate-EDTA (Selander et al.,.1971; Harris and Hopkinson, 1976)
[2]Lithium-hydroxyde (Selander et al., 1971)
[3]Tris-borate-versene-EDTA (Selander et al., 1971)
[4]Citric-acid (Clayton and Tetriak, 1972)
[5]Ridgway (Ridgway et al., 1970)

Slicing and staining the gels

While the gels were running, all solid chemicals, solutions and equipment needed for enzyme staining were weighed, mixed and/or organized. Solid chemicals (see Appendices C and D) were weighed on separate, labeled pieces of paper and stored in containers with a desiccant. Three containers were used to store chemicals depending on storage temperatures (20°C, 4°C, and -20°C). Staining-buffer solutions for each individual enzyme (see appendix B) were mixed in previously labeled plastic beakers (100 ml capacity) and stored at 4°C. Some chemicals were weighed just prior to adding to the staining solution. The staining boxes were labeled with experiment number, the type of buffer system used, assayed enzyme, and the date of the run.

Once electrophoresis was complete, the power supply was turned off and the gels left in the freezer for 5 min. The total running time for each gel was noted. Gels were taken out of the refrigerator and a note was made on the distance migrated by the dye to the nearest mm. Using a blunt scalpel, the gel was trimmed on all sides to facilitate slicing and to fit in the staining boxes (clear styrene, cat. #A401, Flambean Products). A diagonal notch was made on the top left corner to mark the side of the gel with the origin of the sample sequence. The anodal portion of the gel was carefully retrieved from the mold and the filter wicks were removed, making sure no wick pieces were left on the gel. Then the gel was blotted with paper towels, gently set on top of the gel slicer, covered with a long piece of plexiglas and a 2-lbs weight was put on top of it to prevent sliding. With a custom designed "guitar-string slicer", each gel was sliced into several slices (usually six, 1.5 mm slices, plus the top and bottom which were each 1.0 mm thick). Slices were carefully placed in the empty staining boxes, and immediately stained or stored at 4°C until staining. The top and bottom slices are suboptimal, however, they were not discarded until the staining was over in case an extra slice was needed.

The buffer solutions and dry-solid chemicals were mixed in beakers over a magnetic stirring plate right after (or during, if two persons are working together), slicing the gels. After the solids had completely dissolved, the mixture was carefully poured over the gel, and the box was shaken gently to distribute the stain evenly. The gel slices were then incubated at 37°C and monitored regularly until the bands were intense and clear (15 min to 3 hr), at which point they were photographed. Some gels were not incubated but left in a drawer at room temperature (e.g., GPI, see appendix C) to prevent overstaining, which results in unresolvable bands. Most gels were kept in the dark since the three components of the formazan-base dyes (PMS, MTT, NBT) are sensitive to light. When bands were dark and clear, the stain solution was poured out and the gel was covered with the fixing solution (5:5:1 dH_2O:methanol:glacial acetic acid), photographed again, wrapped in plastic wrap, and stored for later scoring or until the photographs were developed. Some enzymes were photographed at intervals during the staining. Others were scored immediately after staining because the bands fade away with time (e.g., ODPH, PGM).

Gel Interpretation and Scoring

Interpretations of the pattern on an allozyme electrophoresis gel after visualization by histochemical staining (i.e., the band pattern comprising the zymogram, see Figs. 2 and 3) require a knowledge of the subunit structure and genetic control of the particular enzyme system. The

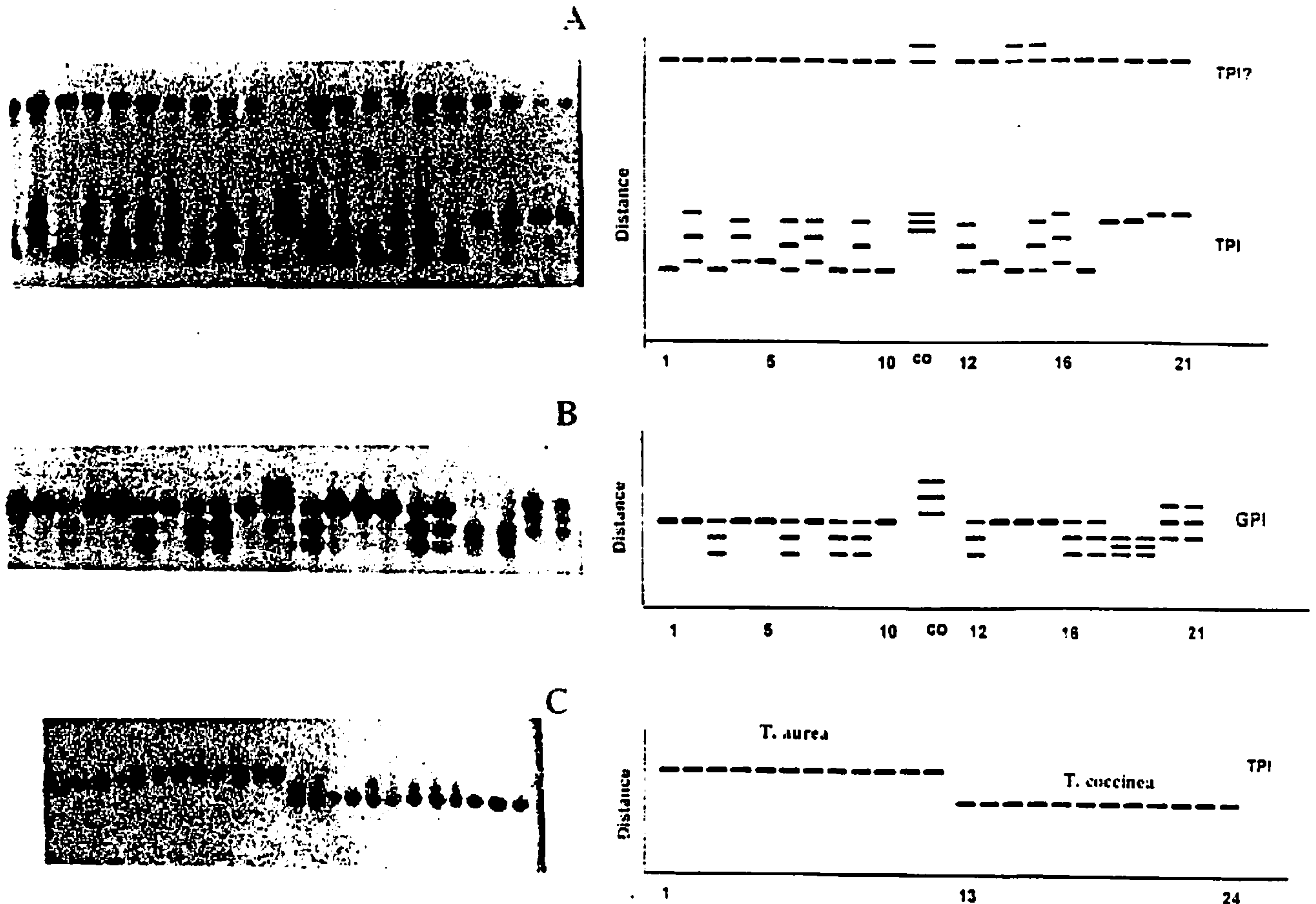

Figure 2. Gel photographs and zymograms showing banding and allele distribution for (A) Triosephosphate isomerase (TPI) and (B) Phosphoglucose Isomerase (GPI) in the ramose *Porites* of the Caribbean [*P.furcata* (1-4,12,13), *P.divaricata* (5-8, 14-17), and *P.porites* (9,10)], and two massive, eastern Pacific *Porites* [*P.lobata* (18,19) and *P.panamensis* from Saboga (20) and Uva islands (21)]. The control (co) is *Tubastrea coccinea* in position 11. In (A) there is banding for two Isomerases; the top one is a polymorphic (n= 2 alleles), unidentified isomerase (TPI?) showing a monomeric structure (two banded heterozygotes). The bottom banding is from one locus of TPI showing 4 distinct alleles and the three banded pattern for heterozygotes of a protein with a dimer structure. (B) shows a polymorphic GPI with heterozygotes (three banded pattern) and homozygotes (one dark band) clearly stained. The banding pattern for TPI in the Caribbean *Tubastraea aurea* (1-12) vs. the Pacific *T. coccinea* (13-24) is shown in (C). Note the fixed, monomorphic pattern of two different alleles in the two species. All loci were consistent with Mendelian models of inheritance at single loci.

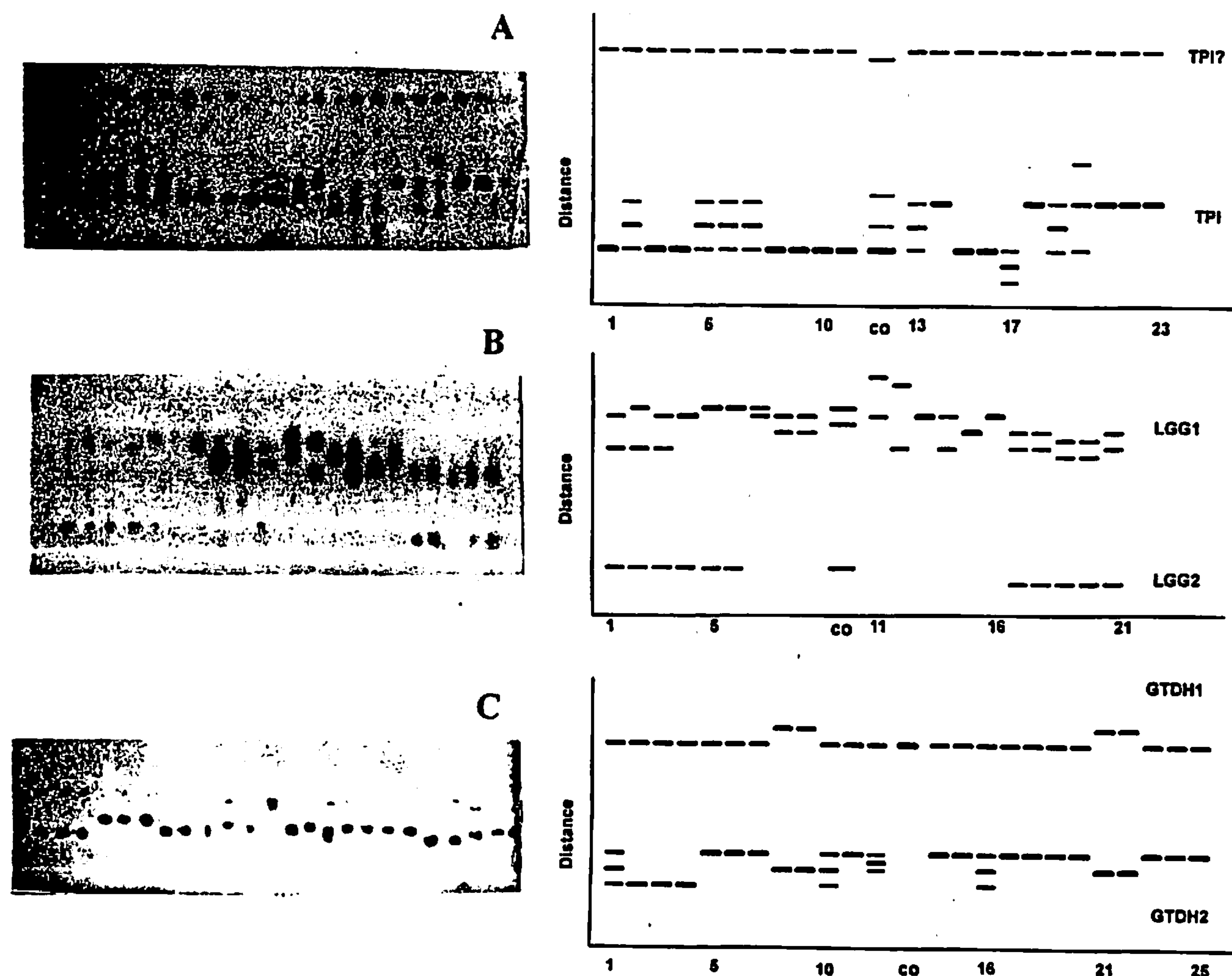

Figure 3. Gel photographs and zymograms showing banding and allele distributions for (A) Triosephosphate isomerase (TPI) and an unidentified monomeric isomerase (TPI?) in *Montastraea cavernosa* (1-11; 13,14), *M. annularis* (16,17,21), and *M. faveolata* (15- 18-20, 22-23). The control in position 11 is *Tubastrea coccinea.* **(B)** The monomer Leucyl-glycine-glycine-peptidase (LGG) for a group of Caribbean agaricids (1-7) and *Manicina areolata* (8-9, 11-16), and the eastern Pacific species *Pavona varians* (17-18-21) and a possible new species of *Pavona* (19-20). Note the faded stain pattern of the bottom locus which makes it subobtimal for consistent scoring. 9 alleles are characterized for the top locus across the 10 species screened in this gel. (C) Two loci of glutamate dehydrogenase (GTDH1, GTDH2) for *Montastraea franksi* (1-4,14-16), *M. faveolata* (5-7,17-19), *M. annularis* (10-12, 22-25), and *M. cavernosa* (8,9,20,21). The top locus is in the limits of resolution for consistent scoring. Bottom locus stained well for all samples, is clearly polymorphic, and is consistent with Mendelian models of inheritance at single loci.

subunit structure of all enzymes screened in this research can be found in Table 3 (see Richardson et al., 1986 and Murphy et al., 1990 for an extended discussion). Allozymes were scored by measuring the distance of the bands representing the different alleles from the origin to the center of the band with plastic vernier calipers. These distances were all related to the control to order the different alleles in each locus. Alleles were labeled alphabetically in order of decreasing electrophoretic mobility from the origin.

Zooxanthellae Contamination

When working with cnidarians, there is always the question of possible contamination with proteins from the symbiotic dinoflagellates (zooxanthellae) inhabiting the endodermal tissue layer, and other organisms like algae and boring sponges living within the skeleton (Willis and Ayre, 1985). To test for contamination from algal proteins, twelve colonies of different species of *Porites* and *Montastraea* were divided into two halves and left to heal for a few days. One half of each colony was placed in a running sea water table covered with two layers of black plastic foil and left in there until the tissue appeared completely bleached (25-35 d at 26-29°C water temperatures). The other half of each colony was kept under ambient light conditions in an adjacent water table. All samples were collected the same day and processed in the same manner. Each pair of tissue homogenates was run side by side in the same gel. No differences were found between the banding patterns of each pair for all the species tested, suggesting that our grinding methods produce no significant contamination with proteins from the dinoflagellated algae. These results agree with those of Stoddart (1983) and Garthwaite et al. (1994). More stringent homogenization methods, like tissue sonication, or prolonged grinding might rupture the algal cells and produce contamination, however, this needs to be tested. Preventing contamination from boring organisms could be avoided by carefully scraping surface tissue without getting to low into the skeleton.

COMMENTS AND TROUBLESHOOTING

Studies in molecular systematics require tissue samples in which the structure and physiologically active state of proteins and nucleic acids is maintained. Different taxa and sample conditions present varying difficulties. Marine organisms, particularly invertebrates, are problematic whenever collecting localities are located far from lab facilities. Keeping animals alive in closed systems for long periods of time is usually difficult. Freezing of samples has proven to be the most efficient method for preserving and storing tissues, but liquid nitrogen or dry ice may not be locally available. Additional problems can be encountered with collecting and transporting the specimens, import and export permits, airline restrictions, etc. Samples stored for a long term may show deterioration of tissue and a decrease in enzyme activity. Deterioration was not observed in our samples during storage periods of over one year in the ultrafreezer, and enzyme activity remain the same after 6 months.

Sampling methods varied from site to site due to the variability and remoteness of the collecting localities. On a few occasions, colleagues kindly collected some of the samples. The following variations in the collection and storing methods were used: (A) A few branches or small pieces of coral (4 x 4 cm) were cut off or drilled from larger samples, tagged, placed in

Table 3. Allozymes from different Caribbean, eastern Pacific and western Pacific corals screened with starch gel electrophoresis. EC = Enzyme Commision numbers, STRUCT = Quaternary structure[1] : M= Monomer; D= Dimer; T= Tetramer; U= Unknown. First buffer in list produced best resolution. Enzyme activity and band resolution: *** = good, ** = good activity but some faint or inconsis-tent bands, * = poor activity or smeared bands were to inconsistent to be reliably scored . Genera with good enzyme activity (AG= *Agaricia*, AC= *Acropora*, GO=*Goniastrea*, LE= *Leptoseris*, MA= *Manicina*, ME= *Meandrina*, MN= *Montipora*, MO= *Montastraea*, PA= *Pavona*, PC= *Pocillopora*, PO= *Porites*, PL= *Platygyra*, SE= *Seriatopora*, TU= *Tubastrea*). ( ) = former abbreviation of enzyme. Starch gel concentrations and buffer's pH vary across studies (see text).

ENZYME	ABBREVIATION	EC	STRUCT	BUFFER SYSTEMS	ACTIVITY	GENERA	SOURCE
Acid phosphatase	ACP	(3.1.3.2)	M/D	2,4	*, **	PO,TU	k,i
Aconitase hydratase	ACOH(ACON)	(4.2.1.3)	M	2,4	*	PO,TU	m,q,t
Adelinate kinase	AK	(2.7.4.3)	M	1,3	*, **	PO,TU	m,q,t
Alcohol dehydrogenase	ADH	(1.1.1.1)	D	1,3	*	PO,TU	b,c,r
Aspartate aminotransferase	AAT (GOT)	(2.6.1.1)	D	1,2	*	PO,MO,TU	m,q,r,t
Creatine kinase	CK	(2.7.3.2)	D	1,2,5	**	MO,PO,TU	n,r,t
Catalase	CAT	(1.11.1.6)	U	5,2,3	*	PO,TU	l,m,t
Cytosol aminopeptidase	CAP (LAP)	(3.4.11.1)	M	1,6,5	*	AG,AC,MA,ME, MO,PO,PA	l,m,q,t
Esterase	EST1	(Nonspecific)	M	1,6	**	AG,LE,MA,ME, MO,PA,TU	k,l,q,t
	EST2	(Nonspecific)	D	1,6	**	AG,LE,PA,PO,TU	k,l,q,t
Fructose-biphosphate aldolase	FBA (ALD)	(4.1.2.13)	T	2,1	*	PO,TU	l,t
Fumarase hydratase	FUMH (FUM)	(4.2.1.2)	T	2,1,5	*	PO,TU	l,t
Glutamate dehydrogenase	GTDH1	(1.4.1.3)	M	2,1,5	***	AC,MO,PA,PO,TU	a,b,l,m,q,t
	GTDH2	(1.4.1.3)	M	2,1,5	***	AC,AG,MA,ME,LE	a,b,l,m,q,t
General proteins	GP	(Nonspecific)		2	*	MO,PO,TU	q,r,t
Glycerol-3-phosphate dehydrogenase	G3PDH	(5.3.1.9)	D	1,5,3	**	MO,PO,TU	r,s,t
Hexokinase	HK	(2.7.1.1)	M	5,3	*	PO,TU	n,q,t
Isocitrate dehydrogenase	IDH	(1.1.1.42)	D	2,4	*	PO,TU	q,t,m
L-Lactate dehydrogenase	LDH	(1.1.1.27)	T	2,4	**	PO,TU	m,t
Leucyl-tyrosine-peptidase	LTY	(3.4.11/13)	D	5,1,6	***	AC,AG,MA,ME,MO, LE,PA,PO,TU	a,b,c,i,k,l,m, n,o,p,q r,s,t
Leucyl-glycine-glycine peptisase	LGG	(3.4.11/13)	M	5,1,6	***	AC,AG,MA,ME,MO, LE,PA,PO,TU	a,b,c,k,l,m, n,o,p,q,r,s,t
Leucyl-proline-peptidase	LPP	(3.4.11/13)	D	5,1,6	***	AG,MA,ME,MO,LE, PA,PO,TU	a,q,j,l,m,n,p, q,r,s,t
Leucyl-leucine-peptidase	LLL	(3.4.11/13)	T	5,1,6	**	PO,TU	m,t
Leucyl-valine-peptidase	LVP	(3.4.11/13)	U	5,1,6	**	PO,TU	q,t
Malate dehydrogenase	MDH1	(1.1.1.37)	D	2,4	**	AC,AG,MA,ME,MO, PA,LE,PO,TU	a,b,c,g,h,l,n, o,p,q,r,s,t
	MDH2	(1.1.1.37)	D	2,4	***	AG,MA,ME,PA,PO,	g,j,l,p,q,r,s,t

Table 3. Continued.

ENZYME	ABBREVIATION	EC	STRUCT	BUFFER SYSTEMS	ACTIVITY	GENERA	SOURCE
Malate dehydrogenase ($NADP^+$)	MDHP (ME)	(1.1.1.40)	T	2,1	***	AC,MA,ME,MO,PA, PO,TU	l,m,q,r,s,t
Manose-6-phosphate isomerase	MPI	(5.3.8.1)	M	5,3,6	**	PO,MO,TU	a,b,c,g,l,n,p, q,r,s,t
Peroxidase	PER	(1.11.1.7)	U	6,2,1	*	PO,TU	m,t
6-phosphogluconate dehydrogenase	PGDH (6PGH)	(1.1.1.44)	D	2,4,5	**	AG,ME,LE,MO,PA,	g,h,i,j,l,m,p, q,r,s,t
Phosphogluconate dehydrogenase	PGDH (G6PDH)	(1.1.1.44)	D	2,5	*	MO,PO,TU	q,r,t
Phosphoglucomutase	PGM	(2.7.5.1)	M	2,1,6	***	AG,AC,MA,ME,MO, LE,PA,PO,TU	a,b,c,d,g,h,k,l, m,n,o,p,q,r,s,t
Phosphoglucose isomerase	GPI (PGI)	(5.3.9.1)	D	2,4,1	***	AC,AG,MA,ME,MO, PO,TU	a,c,d,g,h,j,l,m, p,q,r,s,t
D-Octopine dehydrogenase	OPDH	(1.5.1.1)	M	1	**	PO,TU,LE,PA,PO,TU	r,t
Super oxide dismutase	SOD1	(1.15.1.1)	D	2,5	**	AG,MA,ME,TU	a,b,c,g,h,j,l
	SOD2	(1.15.1.1)	T	2,5	**	AG,AC,MA,ME,MO, LE,PO,TU	a,b,c,g,h,j,l
Triosephosphate isomerase	TPI1	(5.3.1.1)	M	5,6,1	***	AG,MA,ME,MO,LE, PO	p,q,r,s,t
	TPI2	(5.3.1.1)	D	5,6,1	***	AG,AC,MA,ME,MO, LE,PA,PO,TU	p,q,r,s,t
Xnatine dehydrogenase	XDH	(1.2.1.37)	M/D	4	*	PC,PO	b,l

[1] after Harris and Hopkinson, 1976; Harrison et al., 1986; Murphy et al., 1990.

Buffer Systems:

1 - Tris-borate-EDTA (TVB) (# 6 of Selander et al., 1971)
2 - Tris-citrate (TC8.0) (# 5 of Selander et al., 1971)
3 - Tris-maleate-EDTA (TM) (# 9 of Selander et al., 1971
4 - Citric acid (CT) (Clayton & Tetriak, 1972)
5 - Lithium-hydroxide (LiOH) (# 2 of Selander et al., 1971)
6 - Ridgway (RW) (Ridgway et al., 1970)
7 - Tris borate (TB)
8 - Tris-EDTA (TEC7.9)

Sources: a-Stoddart (1983); b-Stoddart (1984a,b); c- Stoddart (1986); d- Stoddart et al., (1988); e- Heyward and Stoddart (1985); f- Willis, (1990); g- Willis and Ayre (1985); h- Ayre and Resing (1986); i- Ayre and Willis (1988); j- Ayre et al. (1991); k- Hunter (1985); l- Garthwaite and Potts (1988); m- Garthwaite et.al. (1994); n- Miller (1992); o- Stobart and Benzie (1994); p- Knowlton et al. (1992); q- Weil (1992a,b); r- Weil and Knowlton (1994); s- Van Veghel and Bak (1993); t- Weil and Brunetti, unpub.

Table 4. Genetic variability within some species of scleractinian corals from the Caribbean and the eastern Pacific expressed as **%P** - percent of polymorphic loci (99.9 % criterium), **A** - average number of alleles per locus, ***Ho/He*** - mean heterozygosity (direct count)/Hardy-Weinberg expected (Nei's unbiased estimate), **POP** - number of populations (sites) sampled, **N** - number of colonies analyzed.

Species	%P	A	*Ho/He*	POP	N
P. porites	100.0	2.82	0.282/0.459	4	113
P. furcata	90.9	2.82	0.237/0.424	3	108
P. divaricata	90.9	2.45	0.259/0.387	3	51
P. astreoides	100.0	3.64	0.349/0.562	4	176
P. "branneri"	90.9	2.18	0.265/0.340	2	39
P. colonensis	90.9	2.91	0.262/0.406	4	89
P. lobata	90.9	4.36	0.409/0.633	4	100
P. panamensis *	81.8	2.18	0.205/0.318	2	34
P. panamensis **	90.9	2.36	0.236/0.368	1	32
M. annularis	88.9	3.7	0.285/0.380	4	59
M. faveolata	88.9	3.9	0.275/0.374	4	61
M. franksi	77.8	3.1	0.356/0.418	4	58
M. cavernosa	66.7	2.1	0.250/0.310	3	31

*from Saboga island
**from Uva island
data for *Montastraea* species from Weil and Knowlton (unpub. data)

within a genus (e.g., OPDH, SOD, EST, 6PGH, G3PDH, CAP, see Table 2, Fig. 3). Some of these enzymes have been resolved in other studies with corals in the Pacific using gels at lower concentrations (see below), and may be usable in the future for Caribbean specimens after further work to customize the protocols to produce better and more consistent banding patterns.

In the protein electrophoretic studies of western Pacific corals, good resolutions have been reported for the same enzymes stained in this study plus a few others (e.g., NP, SOD, 6PGH and MPI, see Table 2). Although detailed descriptions are generally lacking, some differences in their protocols included lower gel concentrations (11.4-12.0% w/v), buffers with lower pH (e.g., Tris Citrate gels with pH 7.0, and Tris Maleate gels with pH 7.4; Stoddart et al., 1988), and lower electrical power settings (35 mA) than those used in this study (Table 2). In the same instances, the quantity of specific chemicals used in these studies also varied slightly from the amounts specified elsewhere (Selander et al., 1971; Harris and Hopkinson, 1976; Richardson et al., 1986; Murphy et al., 1990). Particular protocols may vary depending on different factors like species under study, starch product, general lab conditions, etc.

The number of loci resolved so far is still low to answer certain evolutionary and phylogenetic questions for scleractinians. However, since allozymes represent good taxonomic characters and only one diagnostic locus is necessary to separate two species (Avise, 1974; Ayala and Powell, 1972; Ayala, 1983, 1984), it is highly likely that at least one locus out of the 9-11 scored would be diagnostic (as defined by Ayala and Powell, 1972) for any given, pairwise

flow, hybridization, and phylogenetic relationships. Given the simplicity, low cost and power of this technique, additional research should be done to develop protocols for more enzyme systems in scleractinian corals in specific and for cnidarians in general.

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LITERATURE CITED

Avise, J.C. 1974. Systematic value of electrophoretic data. Syst. Zool. 23:465-481.

Ayala, F.J. 1982. Population and evolutionary genetics, a primer. Benjamin-Cumming Publishing Co. 288 pp.

Ayala, F.J. 1984. Molecular polymorphism: How much is there and why is there so much. Devel. Genetics, 4: 379-391.

Ayala, F.J. and M. Powell. 1972. Allozymes as diagnostic characters of sibling species of *Drosophila*. Proc. Nat. Acad. Sci. 67:225-232.

Ayre, D.J. and J.M. Resing. 1986. Sexual reproduction of planulae in reef corals. Mar. Biol. 90:187-190.

Ayre, D.J. and B.L. Willis. 1988. Population structure in the coral *Pavona cactus*: clonal genotypes show little phenotypic plasticity. Mar. Biol. 99:495-505.

Ayre, D.J., J. Read and J. Wishart. 1991a. Genetic subdivision within the eastern Australian population of the sea anemone *Actinia tenebrosa*. Mar. Biol. 109:379-390.

Ayre, D.J., J.E. Veron and S.L. Duffy. 1991b. The corals *Acropora palifera* and *Acropora cuneata* are genetically and ecologically distinct. Coral Reefs 10:13-18.

Burton, R.S. 1983. Protein polymorphisms and genetic differentiation of marine invertebrate populations. Mar. Biol. Lett. 4:193-206.

Burton, R.S. 1986. Evolutionary consequences of restricted gene flow among natural populations of the copepod, *Tigriopus californicus*. Bull. Mar. Sci. 39:526-535.

Clayton, J.W. and D.N. Tetrick. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J Fish. Res. Board Canada, 29:1169-1172.

Dessauer, H.C., C.J. Cole and M.S. Hafner. 1990. Collection and storage of tissues. Pages 25-41 *in* D.M. Hillis and C. Moritz, eds. Molecular Systematics. Sinauer Associates, Inc., Sunderland, MA. 588 pp.

Garthwaite, R.L. and D.C. Potts. 1988. Biochemical genetics and phylogenies of *Porites*. 6th Inter. Coral Reef Congress, Australia. Abstracts.

Garthwaite, R.L. and D.C. Potts, J.E.N. Veron and T.J. Done. 1994. Electrophoretic identification of poritid species (Anthozoa, Scleractinia). Coral Reefs. 13:49-56.

Harris, H. and D.A. Hopkinson. 1976. Handbook of enzyme electrophoresis in human genetics. American Elsevier Publishing Company, Inc. New York, NY.

Heyward, A.J. and J.A. Stoddart. 1985. Genetic structure of two species of *Montipora* on a patch reef: conflicting results from electrophoresis and histocompatibility. Mar. Biol. 85:117-121.

Huber, M.E. 1985. Population genetics of eigth species of *Trapezia* (Brachyura: Xanthidae), symbionts of corals.

Hunter, C.L. 1985. Assessment of clonal diversity and population structure of *Porites compressa* (Cnidaria, Scleractinia). Proc. Fifth Inter. Coral Reef Congress, Tahiti. 6:69-74.

Knowlton, N., E. Weil, L. Weigt and H.M. Guzman. 1992. Sibling species in *Montastraea annularis*, coral bleaching and the coral climate record. Science. Vol. 255:330-333.

Lamberts, A.E. 1979. Reef coral identification using protein electrophoresis: a preliminary report. Pages 671-677 *in* D.H. Montgomery, ed. Proceedings of the International Symposium on Marine Biogeography and Evolution in the Southern Hemisphere.

Miller, K.J. 1992. Morphological variation in the scleractinian coral *Platygyra daedalea* (Ellis and Solander, 1786) - Genetically or environmentally determined? Proc. 7th Inter. Coral Reef Symp. Guam 1:550-556.

Murphy, R.W., J.W. Sites Jr, D.G. Buth and C.H. Haufler. 1990. Proteins I: Isozyme electrophoresis. Pages 45-126 *in* D.M. Hillis and C. Moritz, eds. Molecular Systematics. Sinauer Assoc. Inc., Sunderland, MA. 588 pp.

Nichols, E., V.M. Chapman and F.H. Ruddle. 1973. Polymorphism and linkage for mannosephosphate isomerase in *Mus musculus*. Biochem. Genet. 8:47-53

Ohlhorst, S.E. 1984. The use of polyacrilamide gel electrophoresis in coral taxonomy. Paleont. Amer. 54:45-48.

Potts, D.C.V. 1984. Generation times and the quaternary evolution of reef-building corals. Paleobiology 10: 48-58.

Ridgeway, G.J., S.W. Sherburne and R.D. Lewis. 1970. Polymorphism in the esterases of Atlantic herring. Trans. Amer. Fish. Soc. 99:147-151.

Richardson, B.J., P.R. Beverstock and M. Adams. 1986. Allozyme electrophoresis. A handbook for animal systematics and population structure. Academic Press, Sydney.

Rosen, B.R. 1981. The tropical high diversity enigma-The coral's eye view. Pages 103-109 *in* P.L. Forey, ed. Chance, Change, and Challenge. The Evolving Biosphere. London.

Selander, R.K., M.H. Smith, S.Y. Yang, W.E. Johnson and J.R. Gentry. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. Stud. Genet. VI. Univ. Texas Publ. 7103:49-90.

Stevens, P.M. 1991. A genetic analysis of the pea crabs (Decapoda: Pinnotheridae) of New Zeland. Mar. Biol. 108:403-409.

Stobart, B. and J.A.H. Benzie. 1994. Allozyme electrophoresis demonstrates that the scleractinian coral *Montipora digitata* is two species. Mar. Biol. 118:183-190.

Stoddart, J.A. 1983. Asexual reproduction of planulae in the coral *Pocillopora damicornis*. Mar. Biol. 76:279-284.

Stoddart, J.A. 1984a. General structure within populations of the corals *Pocillopora damicornis*. Mar. Biol. 81:10-30.

Stoddart, J.A. 1984b. Genetic differentiation amongst populations of the coral *Pocillopora damicornis* of southwestern Australia. Coral Reefs. 3:149-156.

Stoddart, J.A., R.C. Babcock and A.J. Heyward. 1988. Self-fertilization and maternal enzymes in the planulae of the coral *Goniastrea favulus*. Mar. Biol. 99:489-494.

Van Veghel, M.L.J. and R.P.M. Bak. 1993. Intraspecific variation of a dominant Caribbean reef building coral, *Montastrea annularis*: Genetic, behavioral and morphometric aspects. Mar. Ecol. Prog. Ser. 92:255-265.

Weil, E. 1992a. Genetic and Morphological variation in *Porites* (Anthozoa, Scleractinia) across the Isthmus of Panama. Ph.D dissertation, University Texas at Austin, 238 pp.

Weil, E. 1992b. Genetic and morphological variation in Caribbean and eastern Pacific *Porites* (Anthozoa, Scleractinia). Preliminary results. Proc. 7th Inter. Coral Reef Symp. Guam. 2:643-656.

Weil, E. and N. Knowlton. 1994. A multi-character analysis of the Caribbean coral *Montastraea annularis* (Ellis and Solander, 1786) and its two sibling species *M. faveolata* (Ellis and Solander, 1786) and *M. franksi* (Gregory, 1895). Bull. Mar. Sci. 55:151-175.

Willis, B.L. 1990. Species concepts in extant scleractinian corals: considerations based on reproductive biology and genotypic population structures. Syst. Bot. 15:136-149.

Willis, B.L. and D.J. Ayre. 1985. Asexual reproduction and genetic determination of growth form in the coral *Pavona cactus*: biochemical genetic and immunogenic evidence. Oecologia 65:516-525.

Appendix A. Extractant-grinding buffers tested (Stoddart's buffer worked better and was used in all runs).

Harris and Hopkinson (1976). For 100 ml of buffer:

Sucrose	25.0 g
2-Phenoxyethanol	2.0 ml
Mercaptoethanol	2.0 ml
ddH_2O	96.0 ml

Ohlhorst (1985). For 100 ml of buffer:

Sucrose	8.5 g
KH_2PO_4	0.23 g
K_2HPO_4	1.4 g
Phenoxyethanol	1.5 ml
ddH_2O	98.5 ml

Stoddart (1983). For 100 ml buffer:

Sucrose	10.0 g
NADP	25.0 mg
EDTA	1.0 g
Mercaptoethanol	1.0 ml
ddH_2O	98.0 ml

Appendix B. Protocols to prepare different volumes (1, 2 or 4 *l*) of buffer solutions for electrophoresis of scleractinian corals. Gel-buffer solution used for preparing the gel. Electrode-buffer solution in the tray of the electrophoresis apparatus. Doubled distilled water was used in all solutions.

1 - **LITHIUM HYDROXIDE (LIOH)** (pH 8.4) (Selander et al., 1971, modified by Harris and Hopkinson, 1976).

LIOH-A (pH 8.1)	**1 *l***	**2 *l***	**4 *l***
LIOH (g)	1.2	2.4	4.8
BORIC ACID (g)	11.89	23.78	47.56
LIOH-B (pH 8.4)			
CITRIC ACID (g)	1.6	3.2	6.4
TRIS (g)	6.2	12.4	24.8
NAOH (10 M) (ml)	0.45	0.9	1.8

GEL: 1A:9B
ELECTRODE: Use only buffer A on tray.

2 - **TRIS-CITRATE (TC8.0)** (pH 8.0) (Selander et al., 1971)

ELECTRODE:	**1 *l***	**2 *l***	**4 *l***
TRIS (g)	83.2	166.4	332.8
CITRIC ACID (g)	33.0	66.0	132.0

Use with no dilution.

GEL: Dilute electrode solution in ddH_2O (1:29)

ELECTRODE (ml)	33.3	66.6	133.2
ddH_2O (ml)	966.7	1,933.4	3,866.8

3 - **TRIS-BORATE-VERSENE EDTA** (pH 8.0) (Selander et. al., 1971)

ELECTRODE:	**1 *l***	**2 *l***	**4 *l***
TRIS (g)	60.55	121.10	242.20
EDTA (g)	5.96	11.92	23.84
BORIC ACID (g)	40.19	80.38	160.76

GEL: Dilute electrode solution in ddH_2O (1:19)

ELECTRODE (ml)	43.3	86.6	173.2
ddH_2O (ml)	822.7	1,645.4	3,290.8

Appendix B. Continued.

4 - TRIS-MALEATE EDTA (pH 7.4)

ELECTRODE:	1 *l*	2 *l*	4 *l*
TRIS (g)	12.1	24.2	36.3
Na_2EDTA (g)	3.72	7.44	14.88
MALEIC ACID (g)	11.6	23.2	46.4
NaOH (10 M) (ml)	12.95	25.9	51.85
GEL:			
TRIS (g)	1.21	2.42	4.44
Na_2EDTA (g)	0.372	0.744	1.488
MALEIC ACID (g)	1.16	2.32	4.64
$MgCl_2$-$6H_2O$ (ml)	0.203	0.406	0.82

5 - CITRIC ACID (CT) (pH 6.0/6.1) (Clayton and Tetriak, 1972)

ELECTRODE: (pH 6.1)	1 *l*	2 *l*	4 *l*
CITRIC ACID (g)	8.406	16.81	33.62
GEL: (pH 6.0)			
CITRIC ACID (g)	0.42	0.84	1.68

ADJUST PH OF BOTH SOLUTIONS WITH AMINOPROPYL-MORPHOLINE

6 - RIDGWAY (RW) (pH 8.5) (Ridgway et al., 1970)

ELECTRODE: (pH 6.1)	1 *l*	2 *l*	4 *l*
LIOH (g)	2.52	4.04	10.08
BORIC ACID (g)	18.55	37.10	74.20
GEL:			
RW ELECT. BUFFER (ml)	10.0	20.0	40.0
CITRIC ACID (g)	1.05	2.1	4.2
TRIS (g)	3.63	7.26	14.52
ddH_2O (ml) complete to	1.0 *l*	2.0 *l*	4.0 *l*

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix C. Continued.

TRIS-CITRATE (TC8.0) (Contiinued)

PHOSPHOGLUCOMUTASE (PGM) (EC 5.4.2.2)

+ 0.2 M Tris-HCl (pH 8.0)	4.0 ml
+ 0.1 $MgCl_2$	4.0 ml
+ ddH_2O	20.0 ml
# Glucose-1-phosphate (G-1-P)	5.0 ml
# G-6-PDH	4.0 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

PHOSPHOGLUCONATE DEHYDROGENASE (PGDH or 6-PGDH) (EC 1.1.1.44)

+ 0.2 M Tris-HCl (pH 8.0)	20.0 ml
* 6-phosphogluconate	40.0 mg
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

TRIS-BORATE-VERSENE-EDTA BUFFER (TVB)
(Selander et al., 1971)

Gel Size: 500 ml
Gel Conc : 15.0%
Running time: 6-80 h

pH: 8.0
Power: 50 mA

D-OCTOPINE DEHYDROGENASE (OPDH) (EC 1.5.1.11)

+ 0.2 M Tris-HCl (pH 8.0)	20.0 ml
# Octopine	75.0 mg
NAD	2.5 ml
MTT	2.0 ml
PMS	0.5 ml

CYTOSOL AMINOPEPTIDASE (Former LAP) (CAP) (EC 3.4.11.1)

+ LAP Buffer	25.0 ml
LAP Substrate	1.0 ml

INCUBATE 35 MIN.

* FB-K SALT	75.0 mg

CONTINUE INCUBATION

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix C. Electrophoresis running conditions and staining protocols for the coral allozymes with better activity in each gel-buffer system. All gel slices incubated at 37°C unless otherwise specified.

TRIS-CITRATE (TC8.0)
(Selander et al., 1971, modified by Harris and Hopkinson, 1976)

Gel Size: 500 ml
Gel Conc: 15.0%
Running time : 4-5 h

pH: 8.0
Power: 80 mA

MALATE DEHYDROGENASE (MDH) (EC 1.1.1.37)

+ 0.2 M Tris-HCl (pH 8.0)	20.0 ml
MDH1 substrate-1 (Malate)	10.0 ml
NAD	4.0 ml
NBT	3.0 ml
PMS	0.5 ml

GLUTAMATE DEHYDROGENASE (GTDH) (EC 1.4.1.2)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
* Glutamic acid	300.0 mg
NADP	2.0 ml
MTT	1.0 ml
PMS	1.0 ml

MALATE DEHYDROGENASE-NADP (Former Malic enzyme) (MDHP) (EC 1.1.1.40)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
+ 0.1 $MgCl_2$	3.5 ml
* 1-Malic acid	40.0 mg
NADP	1.0 ml
MTT	1.0 ml
PMS	1.0 ml

GLUCOSE PHOSPHATE ISOMERASE (GPI) (EC 5.3.1.9)

+ 0.2 M Tris-HCl (pH 8.0)	15.0 ml
+ 0.1 $MgCl_2$	5.0 ml
Fructose-6-Phosphate	2.0 ml
# G-6-PDH	1.0 ml
NADP	0.5 ml
MTT	1.0 ml
PMS	0.5 ml

INCUBATE AT ROOM TEMPERATURE

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix C. Continued.

TRIS-BORATE-VERSENE-EDTA BUFFER (TVB) (Continued)

GLYCEROL-3-PHOSPHATE DEHYDROGENASE (G3PDH) (1.1.1.8)

+ 0.2 M Tris-HCl (pH 8.0)	20.0 ml
* F-1-6-Diphosphate	175.0 mg
Aldolase	0.5 ml

INCUBATE 35 MIN.

* Sodium arsenate (arsenic acid)75.0 mg	
NAD	2.5 ml
MTT	2.0 ml
PMS	0.5 ml

CONTINUE INCUBATION

LITHIUM HYDROXIDE (RIDGWAY) (LIOH, RW)
(Selander et al., 1971; Ridgway et al., 1970)

Gel Size: 500 ml	pH: 8.4/8.5
Gel Conc.: 15.0%	Power: 325 mV
Running time: 6-7 h	

ESTERASE-A (EST) (Non-specific)

+ Phosphate A	20.0 ml
+ Phosphate B	4.0 ml
+ ddH_2O	6.0 ml
a-Naphtyl Acetate	1.5 ml
b-Naphtyl Acetate	1.5 ml
a-Naphtyl Propionate	1.5 ml
* FB-RR salt	40.0 mg

ESTERASE-B (EST) (Non-specific)

+ Phosphate A	20.0 ml
+ Phosphate B	4.0 ml
+ ddH_2O	6.0 ml
a-Naphtyl Acetate	1.0 ml
b-Naphtyl Acetate	1.0 ml
a-Naphtyl Propionate	1.0 ml

INCUBATE 20 MIN.

* FB-RR K-SALT	40.0 mg in 10 ml ddH_2O

CONTINUE INCUBATION

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix C. Continued.

LITHIUM HYDROXIDE (RIDGWAY) (LIOH, RW) (Continued)

TRIOSE-PHOSPHATE ISOMERASE (TPI) (EC 5.3.1.1)

+ 0.2 M Tris-HCl (pH 8.0)	5.0 ml
+ ddH_2O	20.0 ml
* EDTA	20.0 mg
* Arsenic acid	230.0 mg
# DHAP	3.0 ml
# G-3-PDH	3.0 ml
NAD	3.0 ml
MTT	1.0 ml
PMS	0.2 ml

LEUCYL-TYROSINE PEPTIDASE (LTY) (EC 3.4.11/13)

+ Peptidase buffer	25.0 ml
* Leucyl-tyrosine	10.0 mg
* Dianisidine	5.0 mg
* l-Amino oxidase	5.0 mg
* Peroxidase	10.0 mg

LEUCYL-PROLINE PEPTIDASE (LPP) (EC 3.4.11/13)

+ Peptidase buffer	25.0 ml
* Leucyl-Proline	20.0 mg
* Dianisidine	10.0 mg
* l-Amino oxidase	10.0 mg
* Peroxidase	20.0 mg

LEUCYL-GLYCINE-GLYCINE PEPTIDASE (LGG) (EC 3.4.11/13)

+ Peptidase buffer	25.0 ml
* Leucyl-Glycine	10.0 mg
* Dianisidine	5.0 mg
* l-Amino oxidase	5.0 mg
* Peroxidase	10.0 mg

LEUCYL-VALINE-PEPTIDASE (LVP) (EC 3.4.11/13)

+ Peptidase buffer	25.0 ml
* Lucyl-valine	10.0 mg
* Dianisidine	5.0 mg
* l-Amino oxidase	5.0 mg
* Peroxidase	10.0 mg

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix D. Electrophoresis running conditions and staining protocols for other allozymes screened from coral tissues. All gel slices incubated at 37°C unless otherwise stated.

ACID PHOSPHATASE (ACP) (EC. 3.1.3.2)

+ 0.05 M Acetate buffer (pH 5.0)25.0 ml	
Acid-phosphatase substrate	0.5 ml
* Fast garnet GBC salt	25.0 mg

ASPARTATE AMINOTRANSFERASE (AAT) (EC. 2.6.1.1)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
* R-aspartic acid	100.0 mg
* Alpha-ketoglutaric acid	50.0 mg
* Fast blue-BB salt	75.0 mg
* Piridoxal-5' phosphate	1.0 mg

ADELINATE KINASE (AK) (EC. 2.7.4.3)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
+ 0.1 $MgCl_2$	1.5 ml
* Adenosine-5'-diphsphate (ADP)10.0 mg	
* Glucose	45.0 mg
G-6-PDH	3.0 ml
Hexokinase	1.0 ml
NADP	1.5 ml
NBT	1.0 ml
PMS	0.3 ml

ALCOHOL DEHYDROGENASE (ADH) (EC. 1.1.1.1)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
1:1 Octanol-Ethanol	3.0 ml
NAD	1.0 ml
MTT	0.5 ml
PMS	0.3 ml

ACONITASE HYDRATASE (ACOH) (former ACON) (EC. 4.2.1.3)

+ 0.2 M Tris-HCl (pH 8.0)	10.0 ml
+ 0.1 $MgCl_2$	1.0 ml
0.1 M Cis-Aconitic acid	5.0 ml
Isocitric dehydrogenase (IDH) 3.0 ml	
NADP	1.0 ml
NBT	1.0 ml
PMS	1.0 ml

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix D. Continued.

CREATINE KINASE (CK) (EC. 2.7.3.2)

+ 0.2 M Tris-HCl (pH 8.0)	30.0 ml
+ 0.1 $MgCl_2$	2.0 ml
* Adenosine 5'-diphosphate	20.0 mg
* Glucose	900.0 mg
* Creatine phosphate	20.0 mg
G-6-PDH	6.0 ml
Hexokinase	160.0 units
NADP	2.5 ml
NBT	2.0 ml
PMS	0.5 ml

CATALASE (CAT) (EC. 1.11.1.6)

H_2O_2	4.0 ml
ddH_2O	25.0 ml

LEAVE FOR 1 MIN, POUR OFF, RINSE IN ddH_2O

KI solution	1.5 ml
ddH_2O	100.0 ml
Glacial acetic acid	0.5 ml

POUR ON GEL (be ready to photograph white bands on dark gel).

FRUCTOSE-BISPHOSPHATE ALDOLASE (FBA) (former ALD) (EC.1.2.13)

+ 0.2 M Tris-HCl (pH 8.0)	20.0 ml
* F-1,6 DIP	300.0 mg
G-3-PDH	1.5 ml
NAD	3.0 ml
NBT	2.0 ml
PMS	0.3 ml

FUMARATE HYDRATASE (FUMH) (former FUM) (EC. 4.2.1.2)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
* Fumaric acid	25.0 mg
MDH-2	0.5 ml
NAD	1.0 ml
NBT	0.3 ml
PMS	0.3 ml

L-LACTATE DEHYDROGENASE (LDH) (EC. 1.1.1.27)

+ 0.2 M Tris-HCl (pH 8.0)	30.0 ml
Lithium lactate substrate	6.0 ml
NAD	1.3 ml
NBT	0.3 ml
PMS	0.5 ml

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix D. Continued.

GENERAL PROTEINS (GP) (Non-specific)

+ Amino-black 108 general protein stain	5.0 ml
+ 5:5:1 glacial acetic acid:methanol:H_2O	35.0 ml

Put in tray for GP only, stain for several hours at room temperature, wash-off with 5:5:1 every couple of hours.

HEXOKINASE (HK) (EC.2.7.1.1)

+ 0.2 M Tris-HCl (pH 7.2)	30.0 ml
+ 0.1 $MgCl_2$	1.0 ml
* alpha-D-Glucose	50.0 mg
* ATP	40.0 mg
G-6-PDH	4.0 ml
NAD	2.0 ml
MTT	1.0 ml
PMS	0.5 ml

ISOCITRATE DEHYDROGENASE (IDH) (EC. 1.1.1.42)

+ 0.2 M Tris-HCl (pH 8.0)	10.0 ml
+ 0.1 $MgCl_2$	1.0 ml
Cis aconitic acid	5.0 ml
Isocitrate	3.0 ml
IDH	2.0 ml
NADP	1.0 ml
NBT	1.0 ml
PMS	1.0 ml

LEUCYL-LEUCINE-LEUCINE PEPTIDASE (LLP) (EC. 3.4.11/13)

+ Peptidase buffer	25.0 ml
* Leucyl-leucine	5.0 mg
* Dianisidine	5.0 mg
* l-Amino oxidase	5.0 mg
* Peroxidase	5.0 mg

MANNOSE-6-PHOSPHATE ISOMERASE (MPI) (EC. 5.3.1.8)

+ 0.2 M Tris-HCl (pH 8.0)	5.0 ml
+ 0.1 M $MgCl_2$	1.5 ml
+ ddH_2O	20.0 ml
* Mannose-6-phosphate	75.0 mg
PGI	3.0 ml
G-6-PDH	5.0 ml
NADP	1.0 ml
NBT	1.0 ml
PMS	0.5 ml

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix D. Continued.

PEROXIDASE (PX) (EC. 1.11.1.7)

+0.05 M Na-acetate (pH 8.0)	45.0 ml
0.1 M $CaCl_2$	0.8 ml
3.0% H_2O_2 (hydrogen peroxide)2.0 ml	
* 3-Amino-9-ethyl carbazole dissolved in 7.0 ml dimethyl/formamide	50.0 mg

SUPEROXIDE DISMUTASE (SOD) (EC. 1.15.1.1)

+ 0.2 M Tris-HCl (pH 8.0)	25.0 ml
NAD	0.7 ml
NBT	0.7 ml
MTT	0.4 ml
PMS	0.7 ml

LEAVE IN THE OPEN LIGHT

*** - Prepared in beaker 4-8 hours prior to run and stored at 4°C.**
+ - Weighed and refrigerated or put into freezer.
 # - Weighed or prepared fresh just before using.

Appendix E. Chemicals in solution for the enzyme staining process.

MDH-1	2.0 M DL Malate
MTT (mg/ml)	1.0 g Tetrazolin in 100 ml ddH_2O
NAD (mg/ml)	1.0 g Diphosphopiradine Nucleotide in 100 ml ddH_2O
NADP (mg/ml)	1.0 g Triphosphopiradine Nucleotide in 100 ml ddH_2O
NBT (mg/ml)	1.0 g Nitro Blue Tetrazolium in 100 ml ddH_2O
PMS (mg/ml)	1.0 g Phenazine Methosulfate in 100 ml ddH_2O
G-1-P (0.046 M)	1.7 g Na2-D-Glucose-1-Phosphate.4H_2O in 100 ml ddH_2O
G-3-PDH	50 u in 1.0 ml ddH_2O (max 25-50 ml)
G-6-PDH	100 u in 10 ml ddH_2O
FRUCTOSE-6-P (18.0 M)	273 mg D-Fructose-6-Phosphate in 50 ml ddH_2O
TRIS HCL (0.2 M, pH 8.0)	24.2 g Sigma 7-9 in 1000 ml ddH_2O
$MgCl_2$ (0.1M)	2.03 g $MgCl_2$ in 100 ml ddH_2O
LAP BUFFER (pH 5.2)	24.2 g Tris 23.2 g Maleic acid in 1000 ml ddH_2O, adjust with /2.0 M Tris
LAP SUBSTRATE	1.0 g 1-Leucil(Leucine)-B-Naphthylamide HCL in 40 ml ddH_2O
DL-MALATE (2.0 M, pH 7.0)	268.2 g DL-Malic acid/l.pH/150.0 g NaOH
ACETATE BUFFER (0.005M, pH 5.0) adjust pH with 0.1 N HCL	6.8 g Na Acetate/ 14.8 ml 1 N HCL dilute to 1000 ml
PEP BUFFER (0.2M, pH 7.0)	28.414 g $Na2HPO_4$ (anhyd) in 1000 ml ddH_2O
PHOSPHATE A (0.2 M NaPhosphate, BUFFER monobasic, pH 4.4)	27.6 g $Na_2HPO_4.H_2O$ in 1000 ml ddH_2O
PHOSPHATE B (0.2 M NaPhosphate, BUFFER dibasic, pH 8.7)	53.6 g $Na_2HPO_{4.7}$ H_2O in 1000 ml ddH_2O
α-NAPHTYL	1.0 g of α-Napthyl Acetate in 99 ml acetone β-Napthyl Propionate Acetate in 99 ml acetone
GEL FIXATIVE	(5:5:1) Methyl Alcohol:ddH_2O:Glacial Acetic Acid

Appendix F. Amounts of starch gel (g) needed to prepare different gel sizes at different concentrations.

CONC. (%)	GEL SIZE (ml) 300	400	500	600	700
11	33	44	55	66	77
12	36	48	60	72	84
13	39	52	65	78	91
14	42	56	70	84	98
15	45	60	75	90	105
16	48	64	80	96	112