PHENOTYPIC AND GENETIC VARIABILITY IN THE OCTOCORAL *Plexaura flexuosa*

by
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A thesis submitted as a fulfillment of the requirements for degree of
MASTER OF SCIENCE
IN
Marine Sciences

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2007

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ABSTRACT

Clonal marine organisms exhibit high levels of morphological variation. Morphological variation may be a response to environmental factors but also they can be attributed to genetic factors. In this study, we examined the extensive morphological variation observed in the gorgonian *Plexaura flexuosa* (Lamouroux, 1821), a widely distributed Caribbean octocoral. We quantified differences in morphological traits in natural populations along depth profiles (shallow <5 m and deep >17 m), degree of protection to water motion and sedimentation rates (from inshore, protected and high sedimentation rates to offshore, exposed and lower sediment loads). Also, transplant experiments and genetic analysis were performed to test for possible ecophenotypic and genetic interactions. Significant differences ($P<0.001$) in 14 morphological traits were found among colonies inhabiting 12 locations comprising seven reefs in southwest Puerto Rico. Results from principal component analysis indicated the presence of two groups based on depth distribution, suggesting the presence of two discrete morphotypes (i.e. shallow type <5 m and deep type >17 m). A discriminant function analysis based on a priori univariate and multivariate analyses (which separated the colonies in morphotypes) correctly classified 93% of the colonies for each environment. Light, water motion and sediment transport co-varied along the depth profiles and they might influence the distribution of the two morphotypes. However, the study was not designed to separate the contribution of each variable. Reaction norms of morphological characters of colonies that were reciprocally transplanted showed gradual significant changes through the 15 months of transplantation. Sclerites of shallow water colonies became larger when transplanted to deeper environments and vice versa, but, neither of the two transplanted groups overlapped with the residents' morphology. Genetic analysis of mitochondrial and nuclear genes suggested that such discrete morphology and non-overlapping phenotypic plasticity is correlated with the presence of two independent evolutionary lineages distributed non-randomly in shallow and deep environments. The genetic patterns found in the two sympatrically living morphs may have resulted from 1) secondary contact after populations diverged in allopatry and reproductive isolation machinery developed, 2) by divergence with gene flow through ecological specialization in sympatry or 3) by the poorly understood process of hybridization in anthozoan evolution.

RESUMEN

Los organismos marinos coloniales presentan una gran variedad de formas pese a su dependencia en la reproducción asexual y por ende baja diversidad genética. Esta variedad de formas les permite mantener sus poblaciones y contribuye a su habilidad para adaptarse a nuevos ambientes. En este trabajo, se cuantificó la variabilidad morfológica en poblaciones naturales a través de gradientes ambientales del común y ampliamente distribuido octocoral *Plexaura flexuosa* (Lamouroux, 1821). Al mismo tiempo, se estimo la contribución genética y ambiental de dichas variaciones morfológicas y su importancia en procesos de adaptación y evolución de la especie. Se encontraron Diferencias significativas ($p<0.001$) en los 14 caracteres morfológicos estudiados en las 12 poblaciones analizadas, sugiriendo que hay dos morfologías discretas asociadas a la profundidad (i.e. tipo-somero <5m y tipo-profundo>20m). Diferencias en el movimiento del agua y el transporte de sedimentos en los diferentes lugares de muestreo pueden estar también asociadas a la variación morfológica. Los dos morfotipos fueron correctamente clasificados (93%) en su propio ambiente en el análisis multivariado discriminate basado en los patrones de profundidad. Las normas de reacción de los caracteres morfológicos en las colonias recíprocamente transplantadas mostraron cambios graduales en su tamaño a través del tiempo (15 meses). El tamaño de las espículas de las colonias someras incremento en el tiempo y viceversa, sin embargo nunca se observo un solape con la morfología de las colonias residentes. La distribución discreta de las características morfológicas, así como un patrón rígido de plasticidad fenotípica están altamente relacionados con diferencias genéticas encontradas en el ADN mitocondrial y nuclear. Aun mas, los dos linajes evolutivos no se distribuyen aleatoriamente en los ambientes naturales y posiblemente han desarrollado mecanismos de adaptación a sus correspondientes habitas, consecuentemente disminuyendo su mortalidad en sus óptimos nichos. El patrón genético encontrado en los dos linajes que viven en sympatria pudo haberse debido a: 1) un contacto secundario después de que las dos formas habían divergido y se había desarrollado maquinaria reproductiva independiente, 2) por divergencia en sympatria a través de especialización ecológica o 3) por el pobremente estudiado proceso de hibridación en la evolución de antozoarios.

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To Cielo and Paulina

"If the misery of the poor be caused not by the laws of nature, but by our institutions, great is our sin." *Charles Darwin*

"In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed… It is not the strongest of the species that survives, or the most intelligent that survives. It is the one that is the most adaptable to change." *Charles Darwin*

ACKNOWLEDGMENTS

I greatly thank the people of Puerto Rico for allowing me to carry out my graduate studies in such a beautiful island and in such a warm environment that made me fill like home. I thank Professor Paul Yoshioka for his support from the very beginning, generous financial help, endless opportunities and patience along this path. I thank Dr. Nikolaos Schizas for awaken in me the passion for the study of evolution, his friendship and unconditional help (including his lab) and his tremendous efforts to make me a more competitive and independent researcher. I thank both for their hours of discussion about ecology, evolution and marine biology. I would also like to thank Dr. Ernesto Weil for his comments along this process to improve the research and his always open door to deepen my knowledge about corals reefs. I thank the University of Puerto Rico, Mayagüez campus and the Department of Marine Sciences, which provided me with endless opportunities, including access to small boats and SCUBA diving materials, the help in the construction of the cement panels for the transplant experiment (Richard Velasquez) and the always kindly help of the administrative staff, special thanks to Angel Camacho (Negrito), Godoberto Lopez (Godo), Neftali Figueroa, Zulma Martinez, Lilivette Valle, Monserrate Casiano (Tati), Nereida Santiago from the Caribbean Coral Reef Institute and Yulissa Garcia from the Puerto Rico Sea Grant.

Funds for this project came from Sigma Xi research-in-aid grant, Sea Grant Seed Money, Restoration of Gorgonian populations from the Sea Grant program of the UPRM, awarded to Dr. Yoshioka, the Coral Reef Ecosystem Studies from NOAA (Dr. Yoshioka chapter), the start up fund awarded to Dr. Schizas from NSF-EPSCOR and a Caribbean Coral reef Institute grant awarded to Dr. Schizas. I would like to thank the Departments of Biology (Dr. Monica Alfaro and Maria Mendez) and Chemistry (M. Sc. Ivelisse Padilla and Sandra Zapata), which provided me with teaching assistantship during my graduate education.

Also, I would like to mention the travel financial support of some institutions and people to assist at two specialized courses ("Development of genetic markers" and "The Summer Institute in Statistical Genetics") and to present at three meetings ("Society for Integrative and comparative Biology, Phoenix, AR January 3-8"; "Evolution 2006. State

University of New York at Stony Brook. June 23-27" and Sigma Xi Poster day University of Puerto Rico, Mayagüez. April 6th 2006"), among them are: The National Institute of Health, the Society for Integrative and Comparative biology, the University of Washington, the University of Puerto Rico through the Arts and Sciences Division (Dr. Astrid Cruz), the Puerto Rico Sea Grant (M.Sc. Ruperto Chaparro) and Dr. Yoshioka and Dr. Schizas.

I would also like to thank Dr. Schizas' and Dr. Yoshioka's students (Alex Mercado, Dannise Ruiz, Elizabeth McLean Joselyd Garcia and Hernan Torres), whose unconditional help, discussion, early training in molecular techniques and Octocoral identification was substantial for this achievement. I especially thank Alex Mercado, Diana Beltran, and Elizabeth Mc Lean for their help in the field.

Discussions with Dr. Juan Sanchez from the University of Andes (Colombia), Dr. Catherine McFadden from the Harvey Mudd College, Dr. Howard Lasker from SUNY at Buffalo, Dr. Daniel Brazeau from SUNY at Buffalo, Dr. Mark Holder from University of Kansas enriched my knowledge about octocorals, analysis of genetic data and provide me with useful comments. DNA sequencing was possible through the sequencing and genotyping facility at the University of Puerto Rico Rio Piedras (M. S. Manuel Ramirez and Dania Rodriguez) and the scanning electron micrographs were possible through the Biology Department of the UPRM (M. S. Jose Almodóvar).

Dr. Yoshioka, Dr. Schizas, Dr. Weil, Dr. Croquer and Dr. Machiavelli improved earlier versions of the manuscript and/or suggested further analysis to them all my gratitude.

Last but not least, I greatly thank Diana Beltran to stand by me these years giving me the strength in difficult moments, her love was essential for this cause. I thank Diana, Andres, Pilar, Nazira, Milena, Giovani, Alex, Stacy, Katie, Brandy, Dannise, Joselyd, Rosalinda (UPRRP), Veronica, Marla, Anabella (UPRRP), Michelle, Michael, Tony, Nestor (UPRRP), Hernan and more recently Dihalia, Carmen, Jorge, Sara, Raquel Aldo, Ainoa and others for make me feel comfortable in such a nice environment; my stay in Puerto Rico would be unforgettable thanks to their friendship. Thanks to Cielo Montoya without her support this work would not have been possible. Her inconditional support has been vital in my education.

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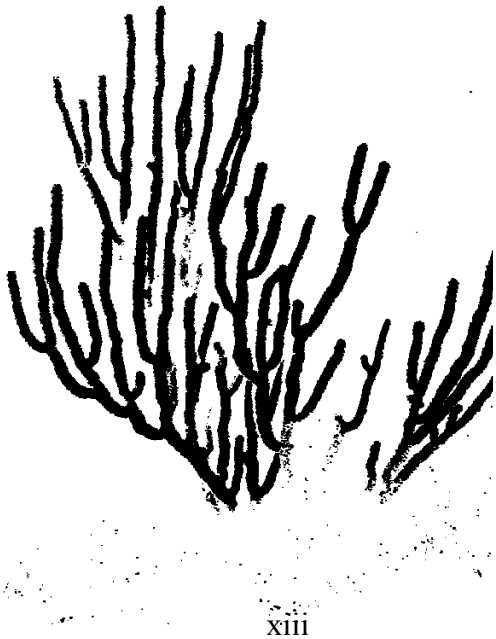

1. INTRODUCTION

Morphological plasticity is important in the maintenance of natural populations, enhancing the survival and reproductive success by contributing to their ability to cope with environmental changes and to potentially adapt to new niches. Despite the importance of plasticity on the fitness of individuals within populations, its implications in the ecology and evolution of species are still uncertain (Schlichting and Pigliucci 1998). The phenotype is the product of genetic interactions and/or responses of the genotype (i.e. norm of reaction) to cope with the environment. Phenotypic plasticity is then the morphological, physiological and behavioral variation due to epigenetic interactions (Futuyma 1998). Also, phenotypic plasticity can act as a balancing effect; generating polymorphism reflected in morphological variation that can sustain environmental changes in the absence of genetic variation. Nonetheless, plasticity is an emergent property of the genotype and therefore also susceptible to natural selection (Pigliucci 2005).

Eco-phenotypic variation is thought to govern the differential responses of a genetically homogeneous population to a heterogeneous environment. The change of the plastic response is often continuous, when the trait (i.e. morphological, behavioral, reproductive, etc.) is analyzed in the environmental gradient suspected to induce the change (Futuyma 1998). This spectrum of phenotypes due to the environmental change describes the norms of reaction (Schlichting 1986; Futuyma 1998). In essence, if genetic interactions are not involved, reciprocally transplanted organisms would eventually develop the native phenotype when submitted to the novel environment.

In marine organisms, transplant experiments have revealed extensive phenotypic plasticity with evolutionary implications. Phenotypic plasticity has been studied in sponges (Palumbi 1984), barnacles (Marchinko 2003), gastropods (Vermeij 1982; Seeley 1986; Trussell 1996; Trussell 2000), bryozoans (Yoshioka 1982; Harvell 1986) and anthozoans (Willis 1985; Willis and Ayre 1985; Gleason 1992; West et al. 1993; Takabayashi and Hoegh-Guldberg 1995; Bruno and Edmunds 1997; Todd et al. 2001; Kim et al. 2004; Todd et al. 2004). If larval settlement in benthic habitats is random, the developing organism has the ability to generate the fittest phenotype suiting local conditions. Morphology is then acquired through development under the current environment and can be changed in the next generation, if conditions are modified. Nonetheless, preferential settlement matching a

genetically constrained phenotype is also possible. Strong environmental gradients in the sea (i.e. light, water flow) may restrict the distribution of individuals to multiple habitats representing opposite ends of the gradient, where each phenotype is adapted (Hilbish 1985; Doebeli and Dieckmann 2003). Furthermore, the fitness of the phenotypes varies along the environmental gradient (Doebeli and Dieckmann 2003). Disruptive selection may enhance the success of the two phenotypes at the opposite ends of the gradient by ecologically favoring each phenotype in its more suitable environment and by increasing genetic divergence. In this case, organisms settle at random in all environments and suffer high mortalities in non-optimal environments. Disruptive selection may be an influential evolutionary force leading to two disparate phenotypes by the existence of non-random mating, due to tight sexual interactions related to habitat utilization (Doebeli et al. 2005).

. Apart from environmental heterogeneity, genetic differentiation can greatly affect morphological variation. First, genes responsible for the morphological change (or those linked to them) can be under selective pressure, changing the frequency of the alleles associated with the trait and generating a selection gradient. Then, differentiated phenotypes could be found at the extremes of the gradient, associated with homozygous individuals at each end of such gradient, while in the contact zone (or hybrid zone) an excess of heterozygotes could be observed.

Second, morphological variation might reflect genetic divergence. Genetically distinct lineages in a geographic area can be explained either by secondary contact after allopatric differentiation has been achieved (Mayr 1963; Coyne 1992); adaptation with gene flow in sympatry (Rice and Hostert 1993) also called ecological speciation (Funk et al. 2002; Avise 2004 for review) or hybridization (Rieseberg et al. 1995; Veron 1995; Arnold 1997).

In marine organisms high dispersal potential of larvae results in genetic homogeneity over large distances (McFadden et al. 1997; Lessios et al. 1998; Grosberg and Cunningham 2001; Lessios and Robertson 2006). However, allopatric speciation has occurred mainly because changes in oceanographic conditions, the emergence of land masses (Knowlton and Mills 1992; Fukami et al. 2004a) or by disconnection of populations by the lowering of sea level (Barber et al. 2002). As gene flow is disrupted by a geographic barrier, populations become isolated, and genes will diverge due to genetic drift. After genetic divergence has

been acquired through generations of genetic drift and restricted gene flow, secondary contact can be achieved when the two new lineages attain similar geographic distributions (Jiggins and Mallet 2000; Mathews et al. 2002). In this case, neutral markers should indicate disruptions of gene flow and the existence of evolutionary distinct lineages.

Apart from allopatric divergence, sympatric divergence is also plausible. Recent studies have shown that speciation occurs in small geographical scales challenging the long standing premises about long distance connectivity in marine systems. The Caribbean contains several species with genetically distinct populations (Taylor and Hellberg 2003; Gutiérrez-Rodríguez and Lasker 2004; Baums et al. 2005; Vollmer and Palumbi 2007). Furthermore, speciation has occurred in spawning organisms with larvae capable of long dispersal (Palumbi and Metz 1991; Knowlton et al. 1992; Weil and Knowlton 1994) and genetic differences have been detected in geographically close populations (Brazeau and Harvell 1994; Carlon and Budd 2002). Ecological specializations to habitats (Knowlton and Jackson 1994; Duffy 1996), symbiotic relationships (Santos et al. 2004), gamete timing release (Lessios 1984; Knowlton et al. 1997; Szmant et al. 1997; Levitan et al. 2004; Fukami et al. 2004b) and a rapid evolution of mating systems (Palumbi and Metz 1991; Palumbi 1994; Metz and Palumbi 1996; Vacquier et al. 1997; Palumbi 1998; Hellberg and Vacquier 1999; Hellberg et al. 2000; Riginos and McDonald 2003; Mah et al. 2005) may prevent organisms to reproduce randomly. Therefore, it is not surprising that sibling species in the sea are more common than previously thought (Knowlton 1993).

Hybridization is another important process of diversification in marine environments. Reticulate and ultimate sympatric speciation can be possible by the production of novel gene combinations in hybrids, therefore increasing phenotypic and genetic diversity in which selection can act. Hybridization and the rise of new lineages due to reticulations is common in plants (Rieseberg et al. 1990; Rieseberg 1991; Arnold 1997). In animals, including marine taxa hybridization is not uncommon. There are instances of hybridization in angelfishes (Pyle and Randall 1994), African cichlids (Schelly et al. 2006), cyprinid fishes (DeMarais et al. 1992; Gerber et al. 2001) and butterflies (Scriber and Ording 2005; Gompert et al. 2006; Mavárez et al. 2006), suggesting that hybridization is an important evolutionary mechanism for speciation in animals. Nonetheless, it is often assumed that discrepancies in phylogenies or of AMOVA results from different molecular

markers is interpreted as incomplete lineage sorting, rather than reticulations as it is in plants (Rieseberg et al. 1990; Arnold 1997). Moreover, in such cases, the interpretation is that one of the study loci (or one linked to it) is under selection to a given environmental feature and the non-differentiation in the other markers is interpreted as incomplete lineage sorting. The phenomenon of reticulate evolution may have great influence in the evolution and diversification of marine species especially those living in sympatry with high potential for hybridization (e.g. spawners). Chromosome incompatibilities or tight gene connections due to rearrangements generated by hybridization can prevent backcrosses, stimulating speciation.

In octocorals, phenotypic plasticity along environmental gradients or habitats is not uncommon (Bayer 1961; West et al. 1993; Brazeau and Harvell 1994; Kim and Lasker 1997; West 1997; Kim et al. 2004; Skoufas 2006). Octocorals are relatively abundant and visually dominant in low relief hard ground habitats with preference for high water motion areas (Kinzie 1973; Lasker and Coffroth 1983; Yoshioka and Yoshioka 1989; Sanchez et al. 1998). Light, water flow and sediment transport are determining factors in the distribution of gorgonians (Yoshioka and Yoshioka 1989). These abiotic factors may induce morphological adjustments in broadly distributed species to optimize fitness under suboptimal conditions.

Furthermore, regional differences may affect species distributions. In the Caribbean, the gorgonians *Plexaura kuna* and *Pseudopterogorgia elisabethae* are abundant in Panama and Bahamas, respectively (Lasker et al. 1996; Gutiérrez-Rodríguez and Lasker 2004), but rare in Puerto Rico (Prada, pers. obs.). Gorgonians are known to exhibit genetic structure in small geographic ranges (Brazeau and Harvell 1994; Gutiérrez-Rodríguez and Lasker 2004; Kim et al. 2004)In *Plexaura flexuosa* Lamouroux, 1821, colonies in shallow fore reefs are susceptible to high water motion and are generally taller with thicker branches and bigger calices. In contrast, colonies in deeper environments are exposed to low water motion and less light. There, the colonies are smaller and not as bushy as their shallow counterparts (Prada, pers. obs.) with smaller and more sparse calices (Kim et al. 2004). Micro-morphologically, sclerite plasticity has also been correlated with differences in water motion and light (West et al. 1993; Kim et al. 2004). Smaller and thinner clubs and spindles are present in high water motion environments (i.e. forereef areas), providing a stronger

structure and support to the colony (West et al. 1993). The high morphological variability in *P. flexuosa* could be due to phenotypic plasticity, genetic differentiation or a combined effect. Consequently, in this study, the morphological variation of 14 traits of *P. flexuosa* was first evaluated in seven reefs (from protected to exposed areas to water motion) at two depths (<5 m and >20 m) and the correlation of morphology with light, water motion and sediment patterns inferred. Second, environmental and/or genetic factors were studied to define the morphological variation of *P. flexuosa*. Specifically, reciprocal transplants of colonies inhabiting the opposite ends of the depth gradient were used to infer patterns, magnitude and direction of the phenotypic response. Analysis of molecular evolution and gene genealogies of the mitochondrial gene msh1 and the nuclear gene 18S were used to elucidate any genetic-phenotypic interaction. Last, the role of allopatric and sympatric divergence (through ecological differentiation) as well as hybridization is considered to explain the genetic and morphological variation found in *P. flexuosa* populations.

2. METHODOLOGY

2.1. *The Species*

Plexaura flexuosa is an octocoral cnidarian forming colonies of about 1m in height in coastal areas, especially coral reefs. The colony is produced by the asexual budding of its polyps, generating a branching dichotomous morphology, arranged in an axial skeleton of fused sclerites of calcium carbonate (Bayer 1961). Each colony is presumably one genotype, unless there has been fusion of larvae during philopatric settlement, as in other modular reef organisms (Jackson and Coates. 1986). Adult individuals exhibit bush-like shape and branch profusely in a single plane (Fig. 1). Microscopically, the apertures (calices) present an inconspicuous lower lip with an unarmed collaret. Sclerites are arranged in three layers. The axial sheath is made up of fused capstans usually purple and ≤200 μm in length. The external layer contains leaf clubs of ~200 μm with 3 or 4 serrate folia and structural spindles (~2000 μm in length) are disposed in a mid layer (Bayer 1961). The last two features distinguish the species from the other plexaurids. For both *P. homomalla* and *P. nina* the leaf clubs and spindles are smaller (150 μm and 700 μm, respectively), spindles are also more slender and calyces usually exerted in *P. nina* (Bayer 1961). Contrary to *P. homomalla*, colonies of *P. flexuosa* in the field usually tend to be branched in one plane but not in a net-like shape with calyces as a lower lip (Sanchez and Wirshing 2005).

FIG. 1. Adult colony of *Plexaura flexuosa* in hard ground bottoms of shallow areas. Adult individuals usually exhibit bush-like shape and branch profusely in a single plane.

In the Caribbean, *P. flexuosa* can be found in coral reefs and tropical rocky walls (Bayer 1961; Muzik 1982; Lasker and Coffroth 1983; Yoshioka and Yoshioka 1989; Sanchez et al. 1998; Sanchez and Wirshing 2005). As other shallow water gorgonians, *P. flexuosa* is relatively abundant in low relief hard ground habitats with preference for high water motion areas (Kinzie 1973; Lasker and Coffroth 1983; Yoshioka and Yoshioka 1989; Sanchez et al. 1998). The distribution of the species spans through several environmental gradients (e.g. depth, light, water motion and sediment transport) (Lasker and Coffroth 1983) and display an unusual amount of morphological variability. For instance, shallow water colonies (typical form *sensu* Bayer 1961) are profusely branched with big and crowded calices and small and concentrated spindles while the deeper water colonies exhibit smaller and less branched colonies with smaller and spaced calices and larger and scarce spindles (Prada pers. obs.).

P. flexuosa is a gonochoric gorgonian that reproduces sexually by spawning gametes (Beiring and Lasker 2000). However, as in other cnidarians, asexual reproduction through fragmentation can take place when loose branches spread to the surroundings (<10m) and new colonies develop from clonemate propagules. However, clonal propagation in *P. flexuosa* is not as common as in *P. kuna* (Lasker 1984). The high density, the widespread distribution in Caribbean reefs, the morphological variation of *P. flexuosa* to environmental changes makes it an ideal system to test hypotheses about the ecological and evolutionary forces that shape the gross morphology of this modular organism.

2.2 *The Environment*

The study was carried out in La Parguera southwest Puerto Rico (Fig. 2) during September 2004 to August 2006. The hydrography of the area has been studied and described elsewhere (Garcia et al. 1998; Morelock et al. 2001). The region is typical of most northern Caribbean reef ecosystems. Reefs off La Parguera are exposed to wave action, generated by the easterly trade winds. The area has the optimum characteristics and water quality for coral reef to flourish. Mean salinity is 35.2 ± 0.92 PSU, while sea temperature is about 28 ± 2.2 °C (Garcia et al. 1998). A warm, dry climate, low wave energy and an extensive insular shelf characterizes La Parguera. The shallow (<10m) benthic community is visually dominated by octocorals, forming colorful gardens, especially in shallow reef platforms with high water motion and wave energy. This development has been enhanced by the die off of the herbivorous sea urchin *Diadema*

antillarum (Yoshioka and Yoshioka 1991). La Parguera is composed by three prominent reef formations, located parallel to the shoreline. 1) Inshore reefs which are more protected to waves and currents, but subjected to higher sedimentation rates, direct contact with sewage discharges and lower scleractinian cover, 2) mid and 3) outer reefs are fully exposed to wave energy, with higher coral cover and significant bottom relief.

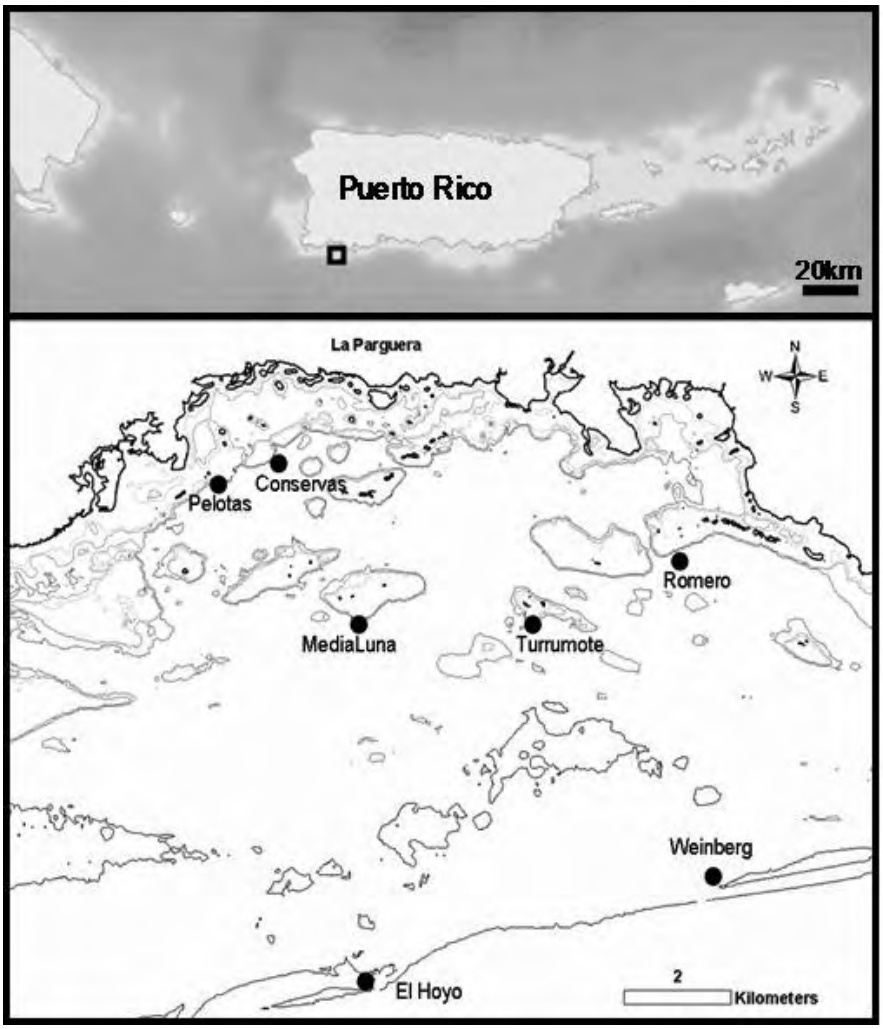

FIG. 2. Top, map of Puerto Rico, indicating La Parguera with a square. Bottom, map of sampling locations in Parguera (Shearer and Nemeth unpub. data).

During the study, colonies in seven reefs were sampled for morphological measurements (Fig. 2). Three inshore, protected reefs (Conservas, Pelotas and Romeo); two mid-shelf reefs

exposed to wave action (Media Luna and Turrumote) and two outer shelf reefs (El Hoyo and Weinberg) were included. In each site, morphological variability of *P. flexuosa* was assessed at two depths (shallow <5m and deep >17m), except in outer reefs (El Hoyo and Weinberg) which are deep (depth 23-27 m). Therefore, in most reefs there are two depths, except the outer reefs. In each location 15 colonies were sampled (n=30/reef). Since water motion can induce morphological variation in *P. flexuosa* (Kim et al. 2004), previous indirect measures of water flow by galvanic corrosion in the area at different depths and reefs were included (McGehee 1998). These results along with other oceanographic conditions of the sampling locations are summarized in Table 1.

Table 1. Relative differences in environmental characteristics of the sampling locations. Estimations on water flow were taken from McGehee (1998); standard deviations for the data were not available. Sedimentation rates are reported as mean values ± standard deviation (Coral Reef Ecosystem Studies project, unpublished data).* Represent not available data for that reef, however the sedimentation patterns are similar to those in Pelotas.

Locations	Coordinates	Zone	Depth (m)	Depth	Water Flow (cm/s)	Sedimentation rates (g/day)
Pelotas	17°57.442 N - 67°04.176 W	Inner	3	PS	4	0.28 (0.18)
			17	PD	9.3	0.11 (0.05)
Conservas	17°57.336 N - 67°02.569 W		3	CS	5.6	*
			17	CD	9.5	*
Romero	17°56.249 N - 66°59.443W		3	RS	> 19.5	0.86 (0.69)
			18	RD	< 13.5	0.34 (0.25)
Turrumote	17°56.097 N - 67°01.130 W	Mid	3	TS	> 25.5	0.46 (0.21)
			17	TD	<14.0	0.15 (0.11)
Media Luna	17°56.093 N - 67°02.931 W		3	MS	> 25.5	0.32 (0.3)
			20	MD	< 14.3	0.15 (0.14)
El Hoyo	17°52.559 N - 67°02.619 W	Outer	22	H	>23.3	0.09 (0.11)
Weinberg	17°53.429 N - 66°59.320 W		23	W	>23.3	0.04 (0.03)

2.3. *Natural variability of morphological traits*

Three branches of the same colony were analyzed to quantify variations among branches within a colony, and between sclerites within branches, prior to testing morphological differences among colonies or among location differences in morphological traits. Colonies grow at the tips, therefore it is possible to find higher number of smaller sclerites in this new tissue than in older tissue. However, new sclerites are produced constantly everywhere in the

colony. Sclerite variation was quantified by measuring width and length of clubs, capstans and spindles at every cm, starting from the tip up to 5 cm baseward (Fig. 3). As only a small fraction of sclerites could be realistically analyzed for length/width variation within a branch, it was critical to determine the branch area at which the spicular analyses would be carried out. The ideal branch sector would be far off the tip of the branch to avoid smaller, underdeveloped sclerites, but allowing new tissue to be generated under the novel environmental conditions during the reciprocal transplant experiment (see transplant experiment section). Individual one-way ANOVAs were used to detect sclerite variation (among branches within colonies and among sclerites within branches).

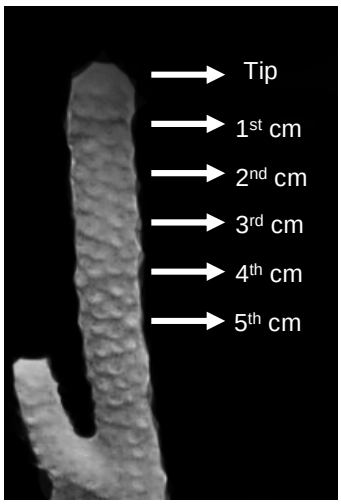

FIG. 3. Graphical representation of the sampling procedure to quantify within branch variation. Each group of sclerites (clubs, capstans and spindles) was sampled at every cm, starting from the tip and baseward.

Three macro-morphological traits: Colony height (CH), branch thickness (BT) and branch development (BD) were measured in 180 colonies, representing fifteen colonies per location (7 reefs per 2 depths, except El Hoyo and Weinberg which are exclusively deep habitats). Branch development was assessed as the total number of terminal branches (Fig. 4b) and the average length of 10 haphazardly chosen branches from branch tip to the first node in terminal branches (Fig.4b).

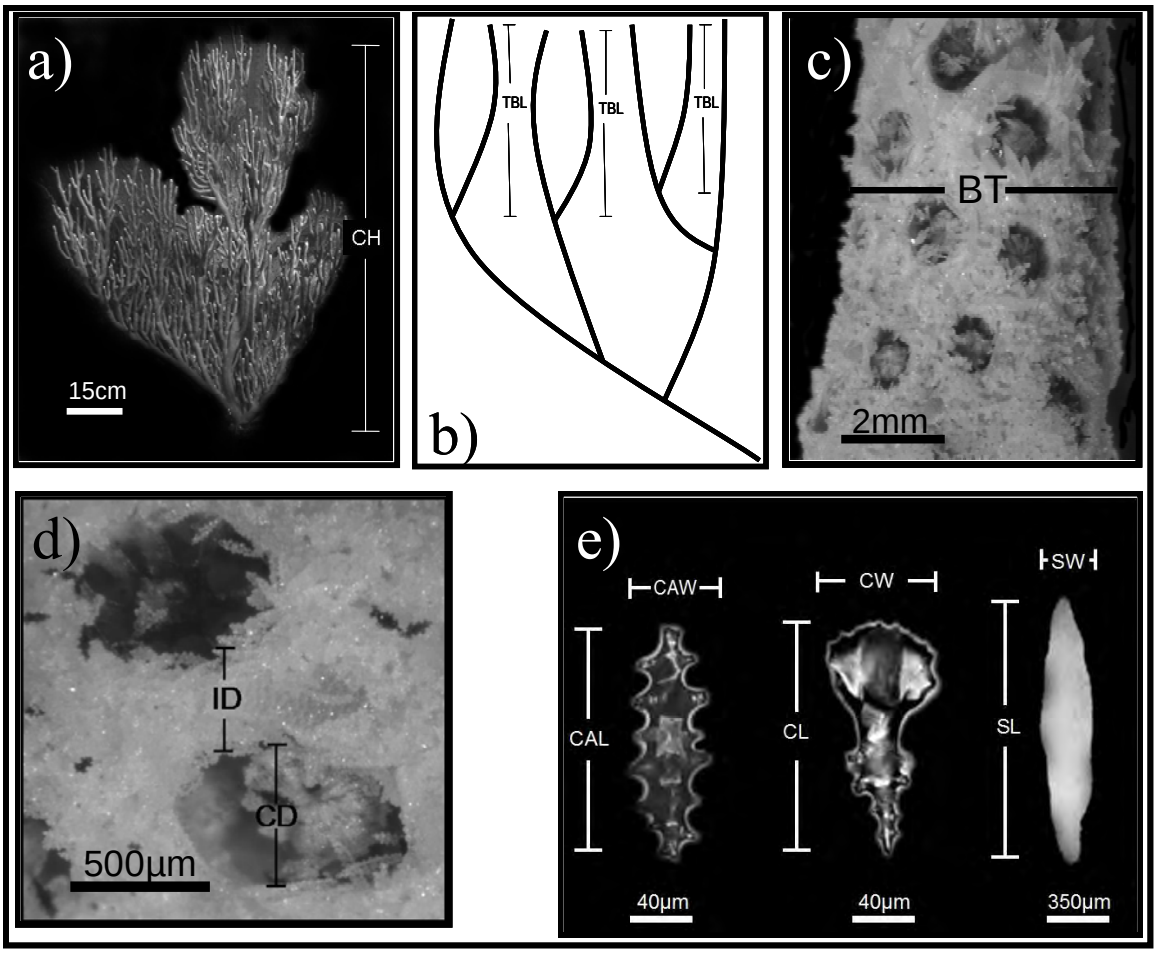

FIG. 4. Phenotypic traits measured in *P. flexuosa*. A) colony height (CH); b) average length of terminal branches (TBL) and number of terminal branches (TBN); c) branch thickness (BT); d) calice diameter (=CD) and intercalice distance (ID) and e) length and width of capstans (CAL and CAW), clubs (CL and CW) and spindles (SL and SW). Also, the surface area in a 1cm length was measured (SA).

Nine microscopic traits were also systematically assessed in each of the same 180 colonies used for macro-morphology. Polyp density (PD), calice diameter (CD), inter-calice distance (ID) and width and length of external clubs (CW and CL) mid-layer spindles (SW and SL) and axial sheath capstans (CAW and CAL) were selected for the analysis (Fig. 4). Polyp density was estimated by counting the number of polyps/cm^2 and standardized with branch thickness, assuming cylindrical shape of the branches. In these micro-morphological traits, 20 measurements (except polyp density) were performed in randomly selected calices in CD and ID or sclerites in CW, CL, SW, SL, CAW and CAL; representing 300 measurements per depth, 600

per reef, 3,600 per character and 28,800 in total, excluding polyp density measures (Table 2). Octocoral branches were collected by clipping off a 5 cm section at the branch tip, slightly bleached with Clorox (5%) to remove some tissue, rinsed in distilled water, and dried. The slight bleaching ensured keeping the colony shape intact and avoiding colony dissolution. For sclerite analysis one cm section at 2 cm from the tip was collected and dissolved with Clorox (5%), following Bayer's protocol (Bayer 1961). For clubs and capstans the samples were taken by placing the spicules in slides and random samples were obtained by moving blindly the slide and measuring all sclerites in each new visual field until 20 sclerites were measured. Spindle, calice and polyp were analyzed by photographing the characters with an Olympus BX-51 compound microscope. All measurements were carried out using photographs (calibrated with a slide of 10 μm accuracy) taken with an Olympus C-5050 camera system attached to an Olympus SZH-10 stereo microscope. Analysis of the photos was performed using SigmaScan (SPSS Inc.).

Table 2. Summary of the characters measured to quantify the morphological variability of *P. flexuosa* in 12 locations (seven reefs) in southwest Puerto Rico. *Terminal branch average length was measured in the first 10 terminal branches whenever possible as there were some colonies with fewer than 10 branches.

Character	**Code**	**Measurements**			
		Per colony	**Per depth**	**Per reef**	**Total**
Club length	**CL**	20	300	600	3600
Club width	**CW**	20	300	600	3600
Ccapstan length	**CAL**	20	300	600	3600
Capstan width	**CAW**	20	300	600	3600
Spindle length	**SL**	20	300	600	3600
Spindle width	**SW**	20	300	600	3600
Calice diameter	**CD**	20	300	600	3600
Intercalice distance	**ID**	20	300	600	3600
Surface area	**SA**	1	15	30	180
Polyp density	**PD**	1	15	30	180
Branch thickness	**BT**	1	15	30	180
Terminal branch number	**TBN**	1	15	30	180
Terminal branch average length*	**TBL**	1	15	30	180
Colony height	**CH**	1	15	30	180

One-way nested ANOVAs (colony nested within reef) per trait were used to test among colony and between group (reef and depth) comparisons. Principal component analysis (PCA) was applied to test the association of the traits and visually examine overall trends, the analysis was done with both raw and standardized (in a 0 to 1 scale) data. The PCA scores of the first

three principal components were used to test for differences among groups and factor interactions in a two-way nested ANOVA. The two factors were degree of protection (zone) and depth (<5m and <20m). Colonies were nested within depths, depth within reefs and reefs within zones. Zone and depth were treated as fixed factors as prior studies have correlated changes in morphology with water motion and depth. The two-way ANOVAs of the first three principal component scores were analyzed in a subset of two inner and two middle reefs. Submerged outer reefs were not included as data of shallow colonies were not available.

Kolmogorov-Smirnov and Levene's test were used to test for normality and homogeneity of variance, respectively (Sokal and Rohlf 1995). Discriminant function analysis (DFA) was used to explore if colonies could be assigned independently to one of the two resulting morphotypes (acquired from the Univariate and PC analysis) and replicate the same predetermined groups in the sampled populations (i.e. zone 1, zone 2, shallow and deep). Wilk's λ was used in the DFA to test for multivariate differences among groups (Quinn and Keough 2002). Individual DFAs were employed for depth, location and zone. The purpose of the DFA analysis was to detect trends in a water motion gradient and/or depth (deep and shallow) on the basis of 14 morphological characters (CH, BT, BD, PD, CD, ID, CW, CL, SW, SL, CAW and CAL).

2.4. *Transplant experiment*

If reciprocally transplanted colonies become similar in sclerite size to the neighboring colonies, the environment is likely to have an effect on the phenotype. As gorgonian growth is slow (<3 cm a year; Yoshioka unpub. data) only sclerite related traits were evaluated. I examined the subapical parts of branches of transplanted gorgonians because this is where new spicules and skeleton material are generated. By avoiding the sampling of tips no underdeveloped sclerites were counted (see within branch variation in the results section).

To test for eco-phenotypic responses of the species to different depth habitats, thirty branches (<30cm) of different colonies separated by >10m from each other, from each depth were reciprocally transplanted in Media Luna reef. Additionally, fifteen branches from each of the two depths were auto-transplanted (transplanted to the same depth) to serve as control. In each depth, 45 branches were transplanted per depth. Fifteen of them were residents and 30 introduced from either shallow or deep, for a total of 90 colonies in the experiment (Fig. 5a).

Each branch was glued into a short piece of plastic PVC pipe with marine hydraulic cement. The cement did not affect the octocoral growth, as live tissue quickly overgrew the dried cement (Fig. 5b); the same material has been successfully used in restoration of gorgonian populations (Yoshioka et al. unpublished data). Each of the plastic pipes was attached to a 1.5 x 1 m cement panel. Fifteen branches were randomly assigned in each cement panel (allowing enough distance to avoid conspecific aggressions) and three panels were placed per depth (45 colonies per depth) separated by 5m from each other (Fig. 5c). Each branch was assigned a code (the same code was also used for genetic analysis) and individually tagged on the panels. The experiments were monitored every month to remove fouling organisms and sediment from the panels. Three times during the 15 months of the study (once every five months) surveys were carried out and linear growth and survivorship recorded. As the transplanted colonies were introduced to a novel environment, the survivorship and linear growth were used to evaluate the overall response (e.g. fitness) of each population. Linear growth was measured from base to tip of the colony and survivorship was recorded as live or dead. Branch thickness and polyp density could also reflect phenotypic plasticity. However, these parameters were not measured because detectable variation would require a longer experimental time due to the slow growth (~3 cm y^{-1}) of *P. flexuosa*.

Linear growth of the transplanted colonies was assessed by subtracting the initial length to the final length. A two-way nested ANOVA was performed to test for differences in population (colonies from shallow and deep areas) and depth (colonies grew in shallow and deep environments) and their interactions. Both factors were considered fixed effects. Chi-square analysis on the number of dead colonies was used to test for survivorship independence among groups. To check for environmental effects on the groups, morphological trends were visually examined by graphing the reaction norm through time per group per trait. As reaction norms plots insinuated a change though time, linear regressions per trait per group was performed to test if the previously identified differences were significant. Also, a repetitive measurement two-way ANOVAs were employed to test the effect of population and time and their interaction. ANOVA assumptions were evaluated as previously described. All statistical analyses were performed in JMP, (version 5.0.1. SAS Institute Inc., Cary, NC), InfoStat version 2004. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) and SigmaStat (SPSS Inc., Chicago, Illinois, USA).

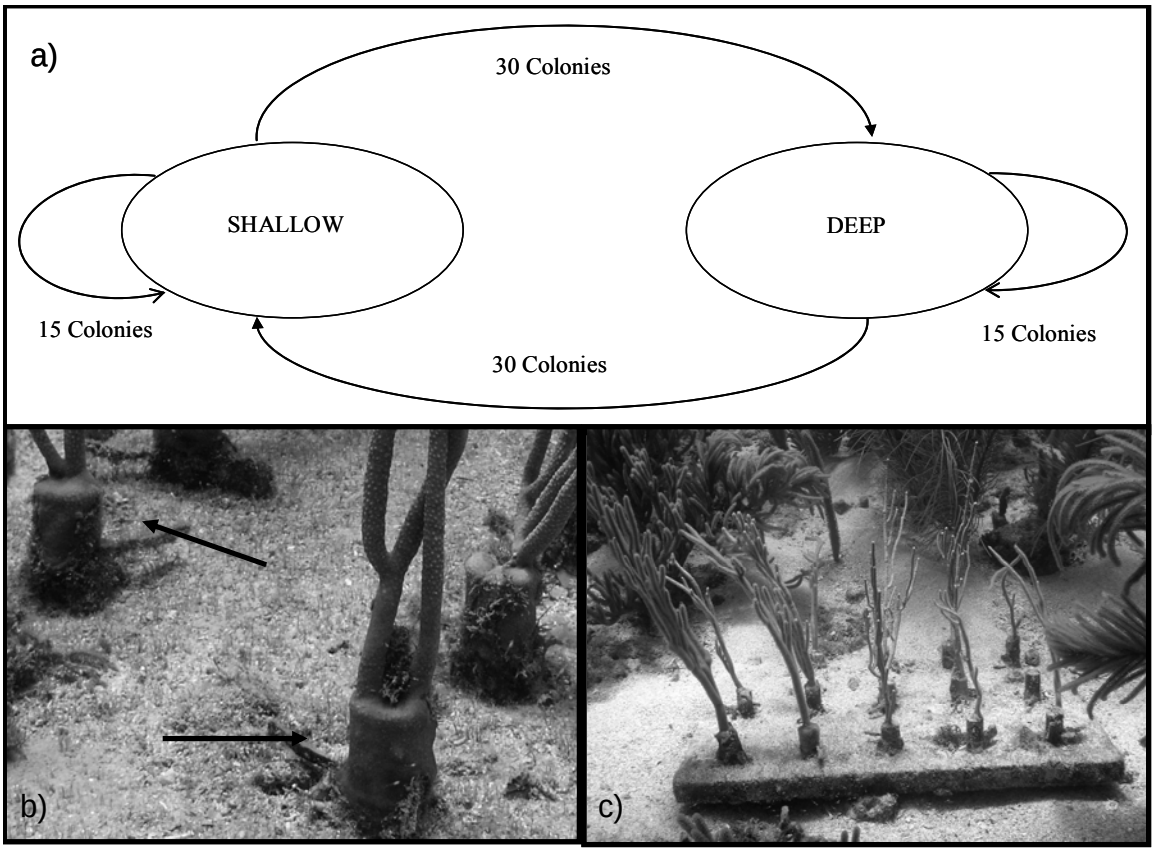

FIG. 5. a) A schematic showing the reciprocal transplant experiment with the controls. b) Arrows showing new tissue overgrowing the plastic tube. No prohibitive effects of the cement or the plastic were recorded during the transplant experiments. c) An *in situ* picture showing a cement panel in which the randomly placed colonies were anchored during the experiment.

2.5. *Genetic Analysis*

The msh1 and the ribosomal 18S genes were sequenced to test for genetic differentiation among populations of *P. flexuosa* living in different depths and reefs (Media Luna, Romero and Isla Culebra) in Puerto Rico. Msh1 is a mitochondrial gene unique to octocorals that codes for a DNA mismatch repair protein and is not present in other Cnidaria (Pont-Kingdon et al. 1995; Culligan et al. 2000). Msh1 and the nuclear 18S provide enough resolution to discriminate between closely related species of octocorals (McFadden et al. 2001; Sanchez et al. 2003; Wirshing et al. 2005; McFadden et al. 2006). If the morphological variation observed in *P. flexuosa* is related to the presence of distinct genetic lineages, morphology will recapitulate the genetic lineages. The msh1 and 18S genes of all transplanted colonies in the eco-phenotypic

experiment were examined, 90 colonies in total (45 individuals for each depth habitat). Additional samples from Romero and Culebra were included in the analysis to test the reproducibility of the preliminary results found in Media Luna. Thirty one colonies from Romero and 21 colonies from Culebra were included for the msh1 analysis. Thirty two colonies from Romero and 21 from Culebra were included for the 18S analysis. A 3-5 cm colony fragment was brought to the laboratory for immediate DNA extraction or stored in 95% ethanol for subsequent work. The PureGene DNA isolation kit (Gentra) for animal fixed-tissue was used for DNA extraction. The polymerase chain reaction (PCR) was employed to amplify a fragment of both genes. The reactions were performed in a volume of 25µl with 1.5 unit of Taq (Eppendorf), 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 10 pmol of each primer, and 50-150 ng DNA. The amplification was performed in an Eppendorf MasterCycler with the same cycling conditions for both genes, consisted of an initial denaturation at 95ºC for 3 min, followed by a touch-down routine of annealing of 10 cycles at 43ºC of 45 sec and 25 cycles at 48ºC of 45 sec; denaturation at 94ºC for 45 sec and elongation at 72ºC for 5 min. The primers used to amplify 18S [A18S (5'-GATCGAACGGTTTAGTGAGG-3') and ITS-4 5'-TCCTCCGCTTATTGATATGC-3')] and msh1 were developed by Takabayashi (1998) and France and Hoover (2001), respectively. Success of PCR reactions was verified by loading a 1% TBE agarose gel with 5µl of the amplicon. Amplified bands were visualized under UV light and recorded digitally. PCR reactions were cleaned from excess dNTPs, primers and other impurities by the enzymatic EXOSAP method. Sequencing reactions were prepared with a DYEnamic ET Terminator Cycle Sequencing Kit (GE). Cycle sequence reactions were loaded in a MEGABase 96 lane Sequencer for capillary electrophoresis. A small portion of sequences was obtained with a 96-lane ABI 377 machine.

DNA sequencing trace files were imported into the Phrap/Phred/Consed programs (Gordon 2004) for base calling, quality assessment, assembly and visualization. Mutations were verified in both the forward and reverse direction. Sequences were then aligned in MacClade (Maddison and Maddison 1992) and compared with BLAST to publicly available sequences of closely related gorgonians. The haplotype (h) and the nucleotide diversity (π), number of segregating sites (S), the Watterson's estimator (θ_w) were evaluated according to Nei (1987) as implemented in DNAsp 4.0 (Rozas et al. 2003). A parsimony haplotype network was constructed for the msh1 sequences using the Templeton et al. (1992) algorithm as implemented in TCS

version 1.21 (Clement et al. 2000). The analysis was conducted using the default settings (95% branch connections). Analysis of molecular variation (AMOVA, Excoffier et al. 1992) of among reefs and between habitats was performed in ARLEQUIN (Schneider et al. 2000). Habitat (shallow and deep) per reef was treated as separate samples and comparisons among them were carried out. Also, group comparisons based on morphological differences (shallow-type and deep-type) were evaluated. The AMOVAs were performed with 10,000 permutations by using conventional F-statistics with haplotype frequencies.

Gene genealogies were constructed for msh1 and 18S using the maximum likelihood (ML) method in PAUP* 4.0b10. MODELTEST 3.6 (Posada and Crandall 1998) suggested that the HKY (Hasegawa et al. 1985) and JC models were the best substitution models for msh1 and 18S, respectively. For the ML analysis, data were bootstrapped 100 times, and sequences were added randomly ten times. Phylogenetic relationships were also constructed using neighbor-joining and maximum parsimony with 1000 bootstrap replicates as implemented in PAUP (Swofford 2002). Given the uncertain phylogenetic position of *P. flexuosa* and its cospecifics (Sanchez et al. 2003; Wirshing et al. 2005), several outgroups were used, including *P. homomalla*, *Muricea muricata* and *Eunicea* spp. Regardless the outgroup, identical topologies were obtained.

3. RESULTS

3.1. *Natural variability of morphological traits*

Comparisons of spicular measurements within branches showed no significant differences in length and width for clubs and capstans. However, spindles at the tip were significantly smaller and narrower than those towards the base. Comparisons of sclerite size among branches within a colony showed no significant differences (Fig. 6). These results suggest that within colony variation is not a major contributor of the variation in sclerite size.

The colonies sampled at different depths and reefs fall within the official description of *Plexaura flexuosa* (*sensu* Bayer 1961). All colonies had big (~ 200 μm) serrate folia clubs, typical of the species (Bayer 1961) and in neither case colonies had an average spindle length of 700 μm (typical of *P. nina* and *P. homomalla*) or slender spindles, armed crown or exerted anthocodiae (typical of *P. nina*). Colonies in shallow areas, would have been undoubtedly identified as *P. flexuosa* proper (Bayer 1961; Sanchez and Wirshing 2005), as they reached about 1 m in height and were dichotomously branched in a single plane with pinhole calices supported by spindles of 1000 μm average length. The 1000 μm spindle length is clearly smaller than Bayer's observations for the species (≥ 2000 μm). Other recent studies have also reported smaller spindles (~1000 μm) for *P. flexuosa* (Kim et al. 2004). The only colonies approximating Bayer's (1961) observations of ≥ 2000 μm in spindle length were found in deep habitats of inshore and mid reefs. The average deep colony of *P. flexuosa* was < 40 cm high, branched not strictly in a single plane and with <20 branches per colony. However, deep colonies found in El Hoyo and Weinberg are either more similar morphologically to shallow water colonies or exhibit an intermediate morphology (Fig. 7, 8, 9 and 10). Mean values per trait and habitat are shown in Table 3. The morphological characters of most colonies in a given location (e.g. Media Luna Shallow) fell within a narrow range around the mean. However, there were some colonies (1 to 3, depending on the location) that did not match the mean population values and were out of the 95% confident level. These atypical colonies were closer morphologically to colonies inhabiting the opposite depth habitat than to their neighbors and showed that in a given location, colonies under similar environmental conditions develop different morphologies. Per trait ANOVAs among habitats and colonies are summarized in Table 4. All traits varied significantly ($P < 0.001$ for most traits and $P < 0.002$ for TBL) between reefs and among depths. CL, CW, CAL, CAW

CD, SA, BT, CH, PD, TBN, and TBM decreased as depth increased. On the contrary, SL, SW and ID increased with increasing depth (Fig. 7, 8, 9 and 10).

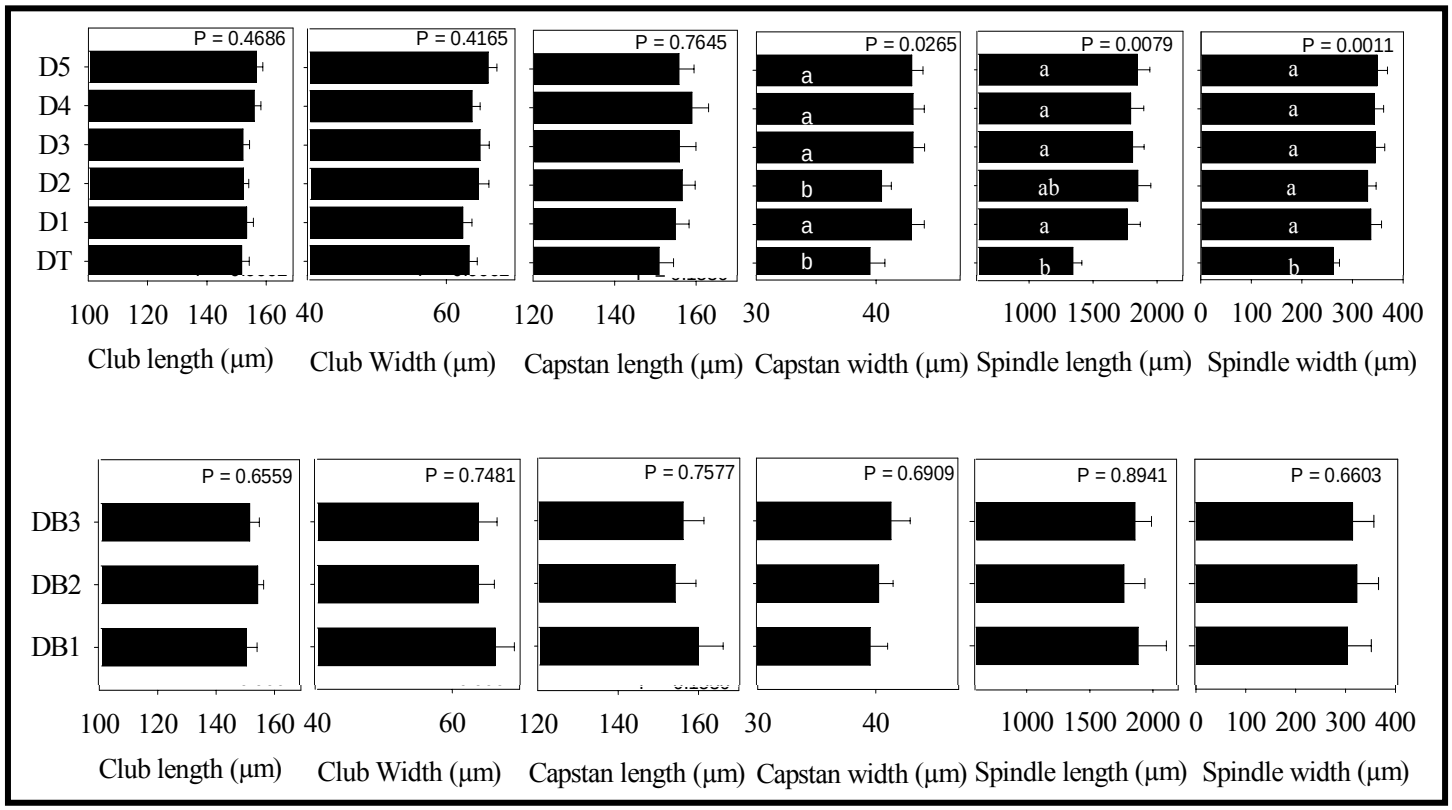

FIG. 6. Variability (mean ± SD) of sclerite traits within branches (top) and between branches (bottom) of in *P. flexuosa*. Numbers in the top figure (within branch variation) represent distance in cm from the tip (i. e. 2 contains sclerites found at 2cm off the tip). Numbers in the bottom represent different branches from the same colony (i. e. DB1 shallow branch one). Note that only spindle (width and length) showed significant differences for within branch variation. Spindles at the tip were smaller than the others.

Table 3. Mean values and standard error (n =300 for micromorphological characters and n = 15 for colony level characters) of *P. flexuosa* morphological characters measured in 12 locations (seven reefs). The location codes are: Pelotas shallow (PS), Pelotas deep (PD), Conservas shallow (CS), Conservas deep (CD), Romero shallow (RS), Romero deep (RD), Turrumote shallow (TS), Turrumote deep (TD), Media Luna shallow (MS), Media Luna deep (MD), El Hoyo (H) and Weinberg (W). The character codes are: club length (CL), club width (CW), capstan length (CAL), capstan width (CAW), spindle length (SL), spindle width (SW), calice diameter (CD), intercalice distance (ID), surface area (SA), polyp density (PD), branch thickness (BT), terminal branch number (TBN) terminal branch average length (TBL) and colony height (CH).

TRAIT	**CD**	**CS**	**H**	**MD**	**MS**	**PD**	**PS**	**RD**	**RS**	**TD**	**TS**	**W**
CL (µm)	148.37 ± 1.37	152.96 ± 1.61	153.03 ± 1.51	159.97 ± 1.86	156.91 ± 1.68	156.96 ± 1.40	157.06 ± 1.76	158.82 ± 1.37	155.39 ± 1.51	143.77 ± 1.46	159.19 ± 1.77	156.20 ± 1.44
CW (µm)	62.72 ± 0.70	63.61 ± 0.82	60.84 ± 0.75	63.11 ± 0.75	64.22 ± 0.89	61.40 ± 0.67	68.48 ± 0.92	65.69 ± 0.72	61.65 ± 0.77	58.79 ± 0.71	64.44 ± 0.96	60.92 ± 0.79
CAL (µm)	148.24 ± 2.69	157.61 ± 2.77	139.35 ± 2.48	151.09 ± 2.86	153.78 ± 2.43	167.37 ± 3.01	175.37 ± 2.59	158.15 ± 2.42	152.61 ± 2.60	144.16 ± 2.62	161.53 ± 2.63	156.81 ± 2.49
CAW (µm)	41.72 ± 0.53	46.65 ± 0.60	44.57 ± 0.49	39.14 ± 0.51	42.71 ± 0.58	40.56 ± 0.59	43.71 ± 0.54	40.98 ± 0.42	48.36 ± 0.52	40.38 ± 0.52	48.96 ± 0.65	41.15 ± 0.51
SL (µm)	1633.43 ± 43.59	1020.66 ± 25.32	1267.64 ± 42.80	2242.99 ± 56.99	956.21 ± 16.59	2477.01 ± 53.36	1113.66 ± 23.06	2371.69 ± 60.40	1047.44 ± 18.55	2063.41 ± 56.63	1060.88 ± 17.09	1853.99 ± 63.36
SW (µm)	377.39 ± 8.94	254.06 ± 6.81	307.26 ± 8.11	469.06 ± 9.61	253.38 ± 4.91	538.74 ± 8.93	265.45 ± 5.26	497.63 ± 9.84	252.01 ± 5.06	470.89 ± 10.18	256.90 ± 4.33	422.15 ± 11.06
CD (µm)	389.04 ± 5.30	459.84 ± 7.26	507.18 ± 7.19	337.10 ± 5.07	543.50 ± 6.48	330.12 ± 4.48	534.39 ± 6.42	353.12 ± 4.32	554.43 ± 7.34	516.68 ± 7.00	547.98 ± 5.11	453.14 ± 7.00
ID (µm)	922.86 ± 17.07	783.66 ± 14.58	737.50 ± 12.88	957.73 ± 16.14	577.36 ± 12.03	961.18 ± 15.31	591.52 ± 10.66	924.10 ± 16.35	550.96 ± 8.82	868.18 ± 16.52	545.45 ± 9.50	858.69 ± 12.87
BT (mm)	3.26 ± 0.09	4.20 ± 0.12	3.87 ± 0.13	2.93 ± 0.06	3.95 ± 0.10	2.93 ± 0.06	3.95 ± 0.10	3.61 ± 0.17	3.98 ± 0.09	2.88 ± 0.10	3.87 ± 0.13	2.88 ± 0.10
CH (cm)	25.11 ± 2.60	38.17 ± 3.21	48.93 ± 4.46	35.53 ± 2.83	46.43 ± 3.95	24.57 ± 2.34	42.10 ± 4.47	29.63 ± 3.00	52.03 ± 5.34	33.00 ± 2.06	58.53 ± 4.23	39.87 ± 3.51
SA (cm^2)	1.19 ± 0.04	1.60 ± 0.05	1.46 ± 0.06	1.06 ± 0.03	1.49 ± 0.04	1.06 ± 0.03	1.49 ± 0.04	1.35 ± 0.07	1.50 ± 0.04	1.04 ± 0.04	1.46 ± 0.06	1.04 ± 0.04
TBM (cm)	4.13 ± 0.45	5.54 ± 0.28	4.59 ± 0.10	4.89 ± 0.46	4.07 ± 0.16	4.21 ± 0.26	6.31 ± 0.38	4.82 ± 0.34	5.13 ± 0.56	4.48 ± 0.35	5.23 ± 0.31	5.07 ± 0.25
PD (count)	43.11 ± 0.5977	66.69 ± 0.4845	63.79 ± 2.26	40.55 ± 0.26	66.53 ± 0.53	41.79 ± 0.32	67.84 ± 0.62	41.51 ± 0.38	67.49 ± 0.73	40.31 ± 0.40	66.11 ± 0.71	61.48 ± 2.49
TBN (count)	10.4 ± 1.26	36.73 ± 5.32	92.13 ± 10.55	12.267 ± 1.634	100.73 ± 7.93	9.53 ± 1.41	46.93 ± 6.41	9.67 ± 1.93	54.33 ± 5.99	14.87 ± 2.62	116.87 ± 13.98	53.13 ± 4.45

Table 4. Extent of variation in phenotypic traits analyzed by nested ANOVA for each morphological character. Colonies are nested within reef per depths (e.g. Media Luna shallow). The analysis was run on untransformed data. The character codes are: club length (CL), club width (CW), capstan length (CAL), capstan width (CAW), spindle length (SL), spindle width (SW), calice diameter (CD), intercalice distance (ID), surface area (SA), polyp density (PD), branch thickness (BT), terminal branch number (TBN) terminal branch average length (TBL) and colony height (CH).

	Effect	df	MS effect	df error	MS error	F	P value
CL	**Location**	11	6763.755	3420	590.83	11.448	<.0001
	Colony	168	3750.909	3420	590.83	6.3486	<.0001
CW	**Location**	11	1973.168	3420	163.196	12.0908	<.0001
	Colony	168	704.5042	3420	163.196	4.3169	<.0001
CAL	**Location**	11	28855.15	3420	1876.12	15.3802	<.0001
	Colony	168	6401.187	3420	1876.12	3.4119	<.0001
CAW	**Location**	11	3187.395	3420	68.988	46.2023	<.0001
	Colony	168	473.5306	3420	68.988	6.864	<.0001
SL	**Location**	11	1.02E+08	3420	449366	227.6439	<.0001
	Colony	168	3015318	3420	449366	6.7102	<.0001
SW	**Location**	11	3700369	3420	15019	246.3759	<.0001
	Colony	168	112411.9	3420	15019	7.4845	<.0001
CD	**Location**	11	2265844	3420	6300	359.6601	<.0001
	Colony	168	116376.39	3420	6300	18.4726	<.0001
ID	**Location**	11	8315230.7	3420	45916	181.0966	<.0001
	Colony	168	291495.41	3420	45916	6.3484	<.0001
BT	**Location**	11	3.90265	168	0.17508	22.2907	<.0001
PD	**Location**	11	1.83	168	0.01	136.01	<.0001
CH	**Location**	11	1705.1	168	197.44	8.6362	<.0001
SA	**Location**	11	0.701711	168	0.032253	21.7562	<.0001
TBM	**Location**	11	6.29392	168	1.81707	3.4638	0.0002
TBN	**Location**	11	0.44	168	0.01	34.90	<.0001

PCA analysis separated colonies of locations (reef per depth, e.g. Media Luna shallow) in shallow and deep areas, similarly as those shown character by character analysis. The first principal component (PC1) explained 64% of the variance and was characterized by high positive loadings for SL, SW and ID and high negative loadings for CW, CAW, SA and BT (Table 5). PC2 explained 16% of the variance and was characterized mainly by positively weighted CL, CW and CAL and negatively by CD

while. PC3 explained 8% of the variance and was mainly influenced by CL, CH (positively) and SA and BT (negatively). Similar patterns were found when data was transformed to a scale from zero to one to avoid unit variation in the different characters measured (Appendix 1).

Table 5. Results of the. Summary table of the results of the principal component analysis. It is shown the first three principal components, calculated from 14 skeletal traits measured on *P. flexuosa,* collected from the 12 locations (seven reefs were included). The character codes are: club length (CL), club width (CW), capstan length (CAL), capstan width (CAW), spindle length (SL), spindle width (SW), calice diameter (CD), intercalice distance (ID), surface area (SA), polyp density (PD), branch thickness (BT), terminal branch number (TBN) terminal branch average length (TBL) and colony height (CH).

Characters	PC1	PC2	PC3
Eigenvalue	7.67	1.87	0.93
Explained Variance (%)	64	16	8
CL	0.14	0.43	0.60
CW	0.26	0.36	-0.07
CAL	0.17	0.53	0.01
CAW	0.27	-0.10	-0.01
SL	-0.31	0.18	0.19
SW	-0.31	0.13	0.16
CD	0.24	-0.37	-0.04
ID	-0.31	0.13	-0.15
SA	0.30	0.09	-0.27
BT	0.30	0.09	-0.27
TBM	0.20	0.32	-0.21
TBN	0.27	-0.23	0.42
CH	0.27	-0.17	0.43
PD	0.32	-0.01	-0.05

PC1 (Fig. 11), resembles the positions of reefs given the degree of protection (from protected to exposed) and the depth profiles, having at the extremes PD (low water motion) to TS (high water motion). Two- way nested ANOVA on the first three principal component analyses (Table 6) showed significant differences in depth and reef and their interactions in PC1. ANOVA analysis on PC2 loadings showed significant differences in reef and reef x depth interaction, while PC3 showed significant differences among zones. Since eighty percent of the variation was explained by the first two principal components, environmental gradients related to depth, water motion, light and sediment transport

could probably explain a portion of the morphological differences. This assertion was further supported by the significant depth interactions in the ANOVA analyses (depth X reef and depth X zone), highlighting the importance of habitat. However, as these co-vary with depth, it was not possible to quantify their individual effects on the morphological variation of *P. flexuosa*.

Table 6. Two-way ANOVA results showing the effects of depth, zone and the interaction between depth and zone on the first three principal components. The PCA loading scores were obtained from analysis of 12 morphological features of *P. flexuosa* in the sampling environments. Note that only eight of the 12 locations were included to achieve a balanced design. Significant values are represented by ***, ** and * for $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Parameter	df	PC1		PC2		PC3	
		MS	F	MS	F	MS	F
Depth	1	98.86067	876.792***	0.0029	0.0031	0.0508	0.0803
Zone	1	0.16466	1.460	2.5699	2.7949	43.5216	68.7550***
Zone*Depth	1	1.59080	14.108**	0.0197	0.0214	1.6042	2.5343
Reef (Zone)	2	0.54477	4.831**	3.0241	3.2888*	1.0421	1.6462
Reef*Depth(Zone)	2	2.33300	20.691***	3.6871	4.0098*	0.4219	0.6665
Error	112	0.11275		0.9195		0.6330	

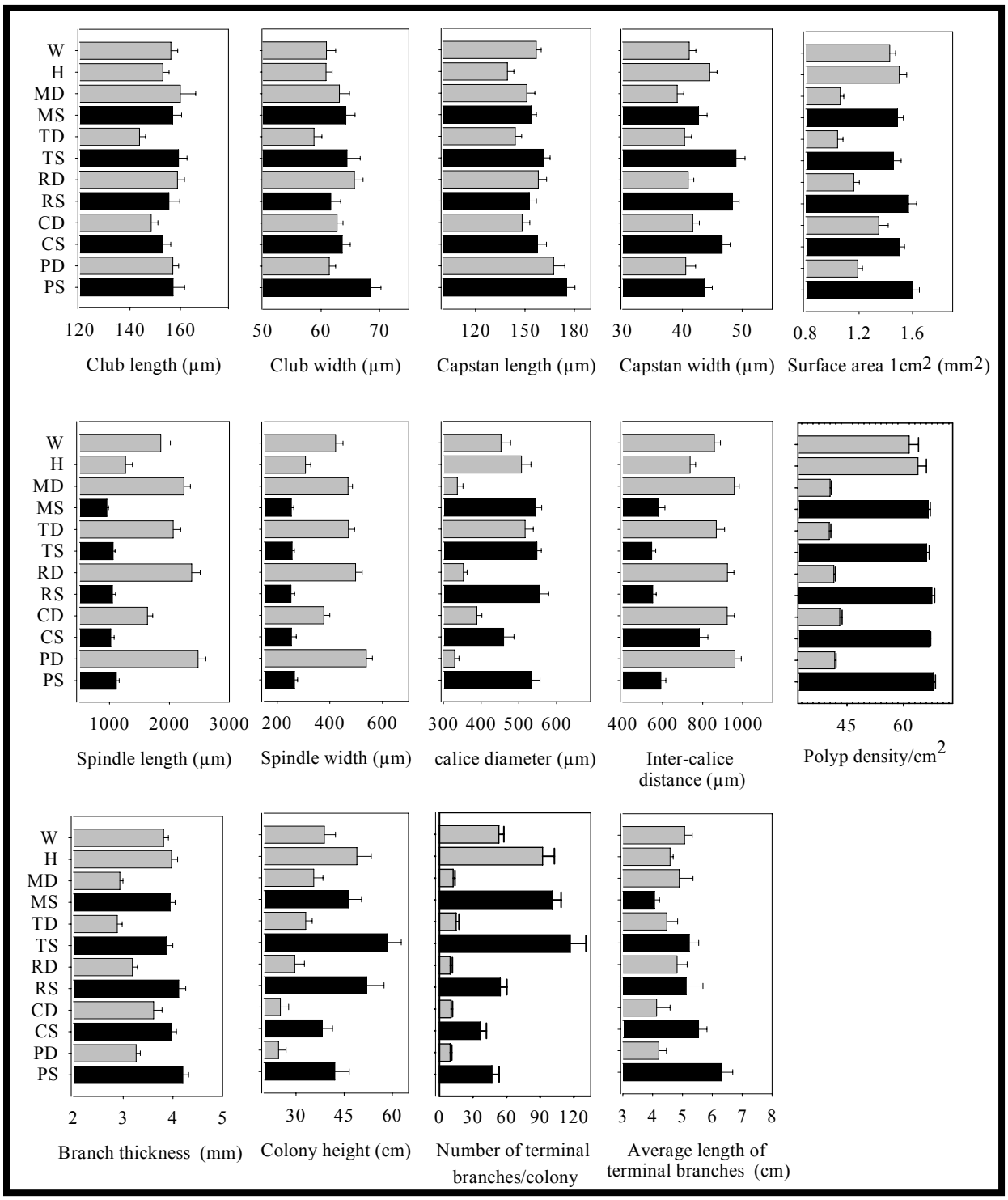

FIG. 7.Extent of variation in phenotypic traits analyzed by individual nested ANOVAs for 12 morphological characters. Colonies are nested within habitats. Values are shown as mean value ±SD. The location codes are: Pelotas shallow (PS), Pelotas deep (PD), Conservas shallow (CS), Conservas deep (CD), Romero shallow (RS), Romero deep (RD), Turrumote shallow (TS), Turrumote deep (TD), Media Luna shallow (MS), Media Luna deep (MD), El Hoyo (H) and Weinberg (W).

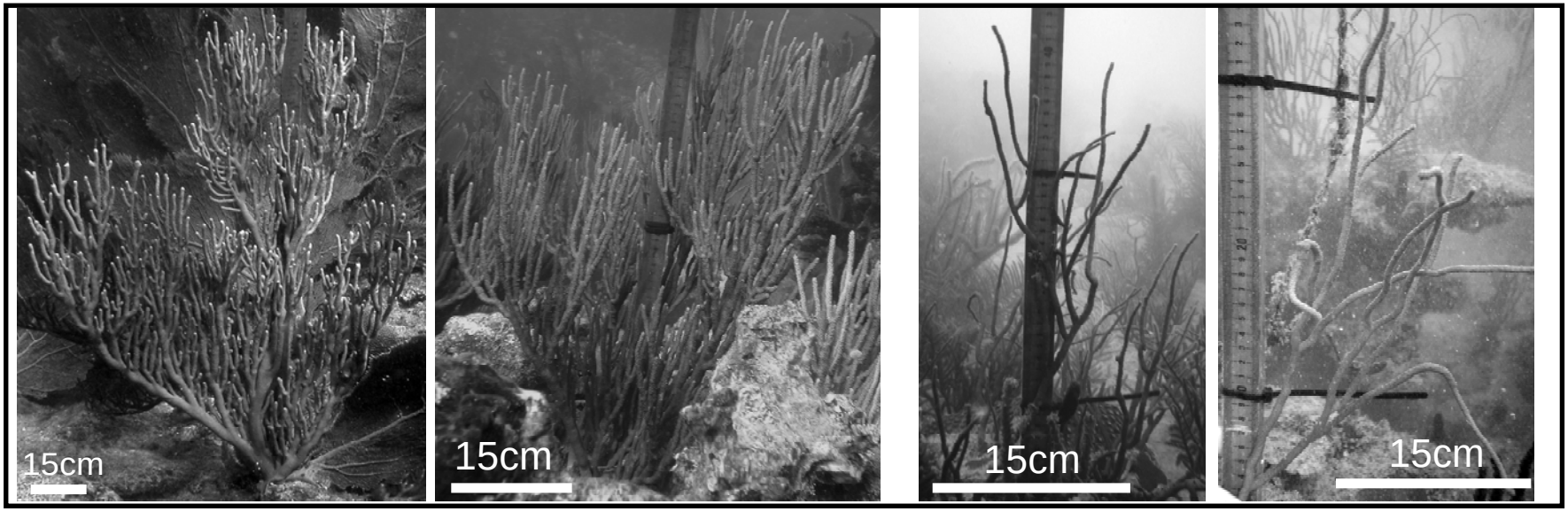

FIG. 8. Differences among colonies inhabiting deep and shallow areas. Colonies in shallow areas (two left), compared to deep colonies (two right) are more branched and bigger in size.

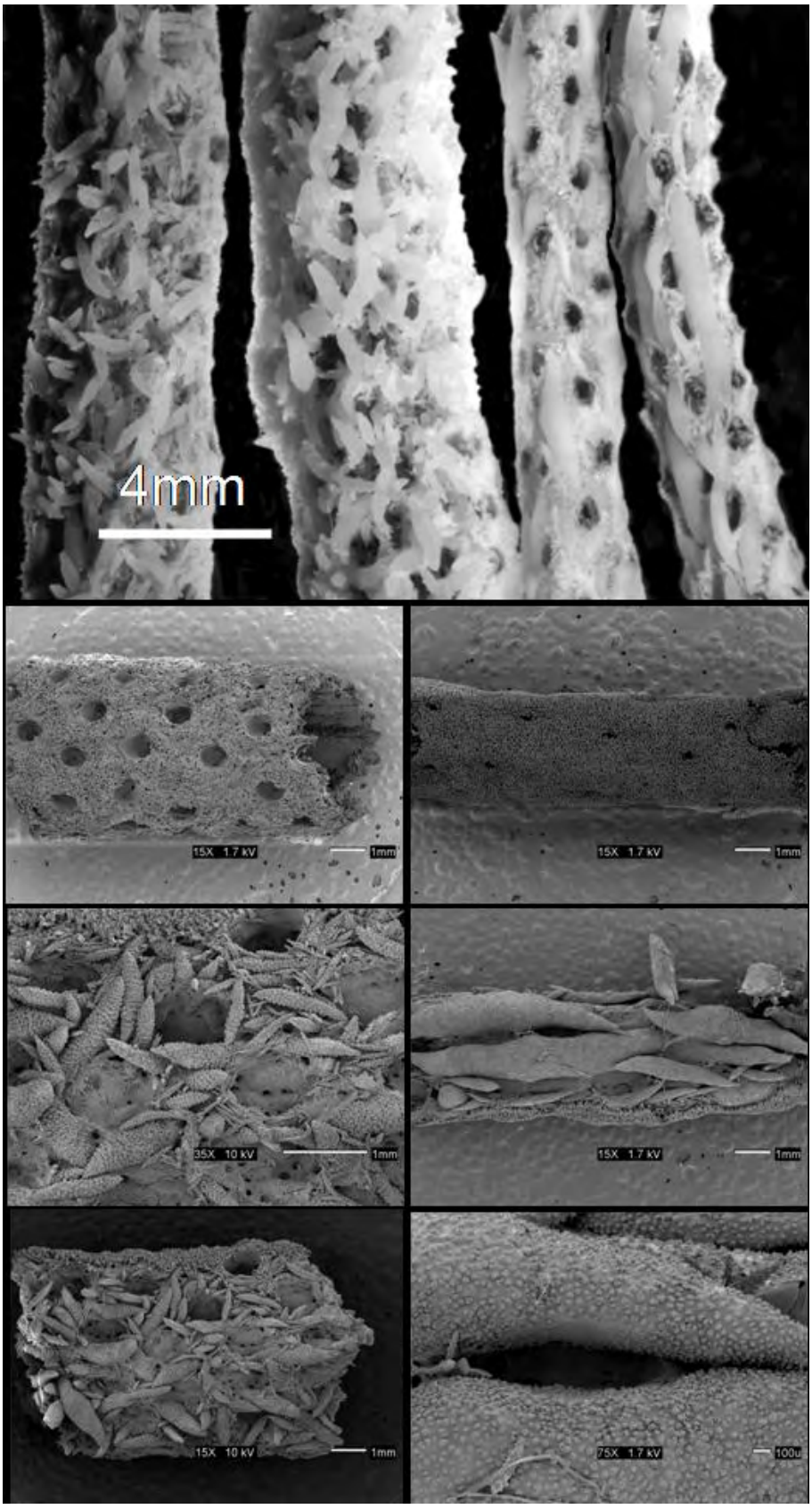

FIG. 9.Branch and calice differences among colonies inhabiting shallow (left) and deep (right) areas. Thicker colonies are found in shallow areas with smaller spindles and more polyps per area.

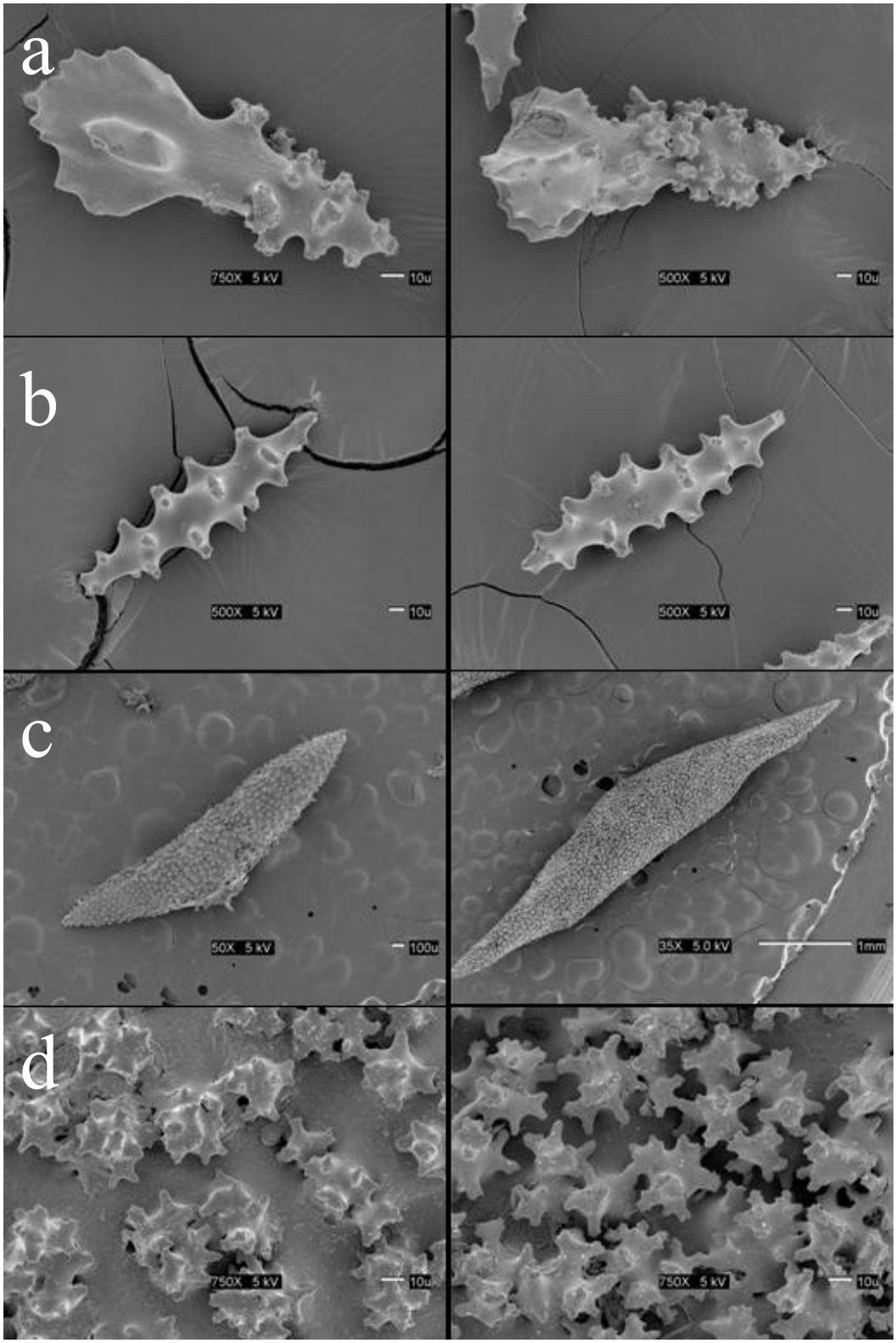

FIG. 10. A) Clubs B) Capstans C) Spindles D) Close up of spindles. Differences among sclerites (club, capstan and spindle) of colonies inhabiting shallow (left) and deep (right) areas. Spindles of shallow area colonies compared to deep colonies are bigger in size. The surface arrangements of warts is sparser in shallow area colonies (shown in D).

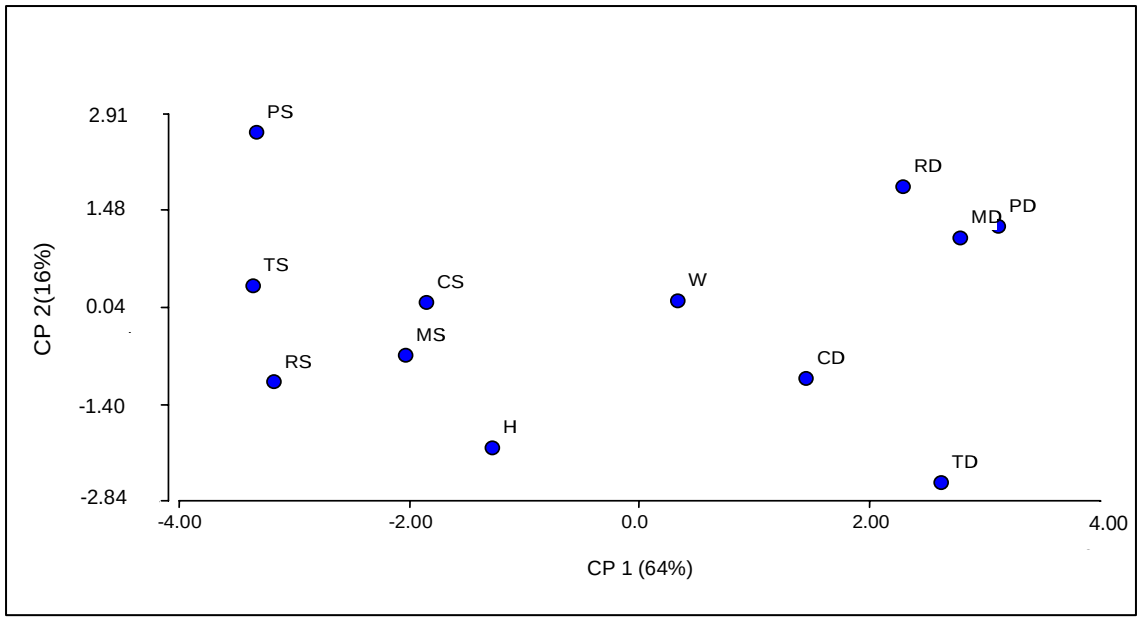

FIG. 11. Principal component analysis of 14 phenotypic traits in 12 locations of *P. flexuosa.* Axes are the first two principal components. The location codes are: Pelotas shallow (PS), Pelotas deep (PD), Conservas shallow (CS), Conservas deep (CD), Romero shallow (RS), Romero deep (RD), Turrumote shallow (TS), Turrumote deep (TD), Media Luna shallow (MS), Media Luna deep (MD), El Hoyo (H) and Weinberg (W). Note that habitats in shallow areas are group on the left of the figure and the deep habitats on the right.

Results of the discriminant function analysis based on Wilk's λ (depth = 0.19, F = 56.29, P <0.0001; reef λ = 0.31, F = 3.60, P <0.0001 and zone λ = 0.21, F = 15.76, P <0.0001) showed significant differences in colonies of different depths, reefs and zones. Depth differences were clearly defined (Fig. 12) and the correct classification percentage was high (93%). The colonies in shallow locations are concentrated on the right side of the canonical plot, while colonies in deep locations are mostly in the left side. There were a few deep water colonies misclassified and embedded within the shallow ones and vice versa (Fig. 12). Also, scatter plots of pairwise traits showed a visual separation of colonies in shallow and deep areas for all trait combinations (Appendix 2 and 3). Canonical plots of the DFAs of reef and zone revealed a gradient from protected and deep to exposed and shallow, however, not further analysis was done as their percentages of misclassification were high at 39% and at 41%, respectively (Appendix 4 and 5).

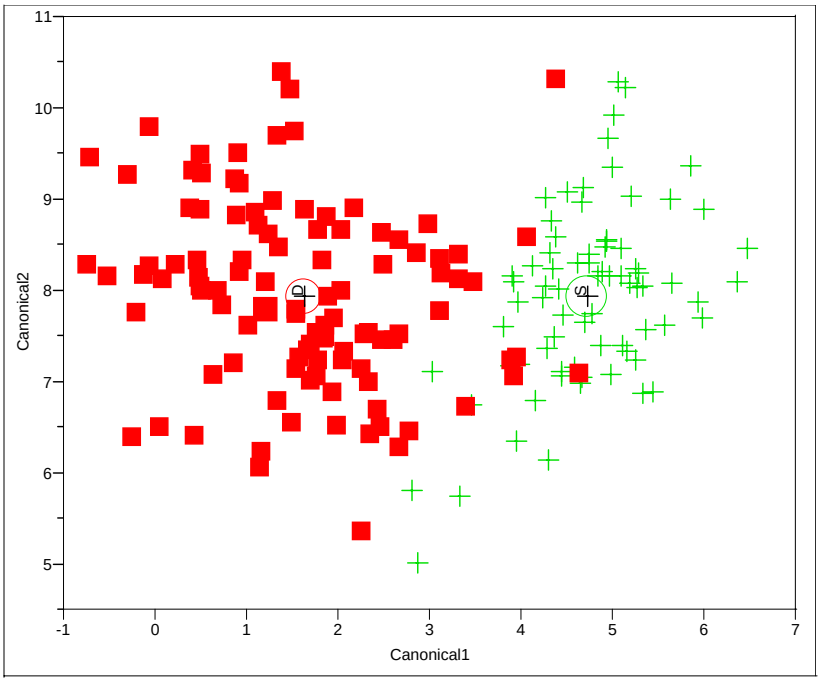

FIG. 12. Discriminant function analysis plot based on 14 morphological characters of *P. flexuosa* colonies inhabiting different depth habitats (D for deep and S for shallow). Multivariate comparison (fixed-effect MANOVA) among depths was significant (Wilks' = 0.2769; F = 30.7713 df = 14/167; P < 0.001). Misclassified colonies = 14 (7%). The canonical axis 1 mostly weighted by length and width of spindle and club, intercalice distance surface area and branch thickness, while canonical axis 2 was mostly weighted by width and length of clubs and calice density.

The univariate and principal component analysis results suggest the presence of two morphological groups (morphotypes) related to different depth profiles. The two morphotypes (the deep-type and shallow-type) were recovered with 93% correct classification by a discriminant function analysis. The two morphotypes are found in two depth habitats with different environmental conditions (e.g. water motion, light, sediment transport). However, each morphotype was recorded in low numbers at the opposite habitat. The shallow-type was found more frequent in deep habitats than the deep-type in shallow areas. Also, colonies sampled in the outer shelf reefs were morphologically closer to those in shallow areas of inshore and mid reefs; however the presence of both morphotypes was higher in such environments and each type was represented by at least 6 colonies (15 colonies total). In Weinberg, the presence of deep-type colonies was associated with sand channels. Along these channels deep-type colonies were more frequently encountered.

3.2. *Transplant Experiment*

3.2.1. *Growth and survival*

On average transplanted branches grew 1.94 ± 0.34 cm (1 SD) during the 15 months of the study. The new tissue deposited at the tip of the branches was enough to allow sampling of sclerites developed under novel conditions [either from shallow to deep (SD) or deep to shallow (DS)], thus the analysis exclude premature sclerites present at the very tip of the colony (see within branch analysis). A two-way ANOVA (Table 7) on linear growth values revealed a significant difference (P = 0.001) across depths (shallow vs. deep). Colonies in shallow environments regardless of population source (residents or transplanted) grew almost twice as fast as their deep counterparts. Population source and population X depth interaction were not significant (Table 7). Of the 90 initial colonies transplanted (30 reciprocal and 15 controls per location), 59 were recovered for sclerite analysis. The mortality was not independent among groups (X^2 = 10.449, df = 3, P < 0.025). The control colonies either from deep to deep, or shallow to shallow had higher survivorship (93% and 80%, respectively) than the transplanted ones. Mortality was highest (57%) in colonies transplanted from shallow to deep areas (Table 8). Most of these colonies died by the 6th month, however neither detachment was noticeable during the experiment, nor there was an obvious presence of predators, nor evident competition with other reef organisms. In most cases the tissue started to fall off the axis until the whole axis was exposed. Colonies transplanted from deep to shallow areas had high survivorship (77%), nonetheless bleaching was recorded in 14 colonies (46%) and 23% of those died.

Table 7. Comparison of linear growth of transplanted colonies two-way ANOVA. The effects tested, were population (residents and transplanted), depth (shallow and deep) and their interaction.

Source of variation	DF	SS	F	P
Population	1	0.168627	0.0668	0.7970
Depth	1	30.667148	12.1479	0.0010
Population x depth	1	0.253637	0.1005	0.7525

Table 8. Mortality rates for the transplanted colonies in number of individuals and as percentage.

	SS	SD	DD	DS
Mortality (percentage)	3 (20)	17 (57)	1 (7)	7 (23)
Total	15	30	15	30

3.2.2. *Transplantation effect*

As predicted from the natural morphological variability analyses, colonies from shallow areas exhibited average spindle width and length (250 and 1000 μm), clubs width and length (65 and 150 μm) and capstans width and length (45 and 155 μm). Colonies from deep areas exhibited average spindle width and length (470 and 2250 μm), clubs width and length (65 and 160 μm) and capstans width and length (40 and 150 μm) (Fig. 13). Gradually, spindle width and length of the reciprocally transplanted colonies became similar to those of the resident colonies but never overlapped, after 15 months of the experiment (Fig 13). Colonies transplanted from shallow to deep areas tent to increase in sclerite size while spindles of colonies from deep to shallow became smaller. CL, CW, CAL and CAW showed similar trends but exhibited lower percent variation within groups, therefore resulting in non-significant (Table 9, 10) between groups comparisons (i.e. DD, SS, SD and DS). However, some colonies (i.e. SD16, SD18, SD19, DS7, DS8, DS14, DS 15, DS17, DS22, DS 23 and DD2) differed significantly from their source population either shallow or deep. Such atypical colonies did not show the gradual change in spicule size over time, observed within their transplanted group.

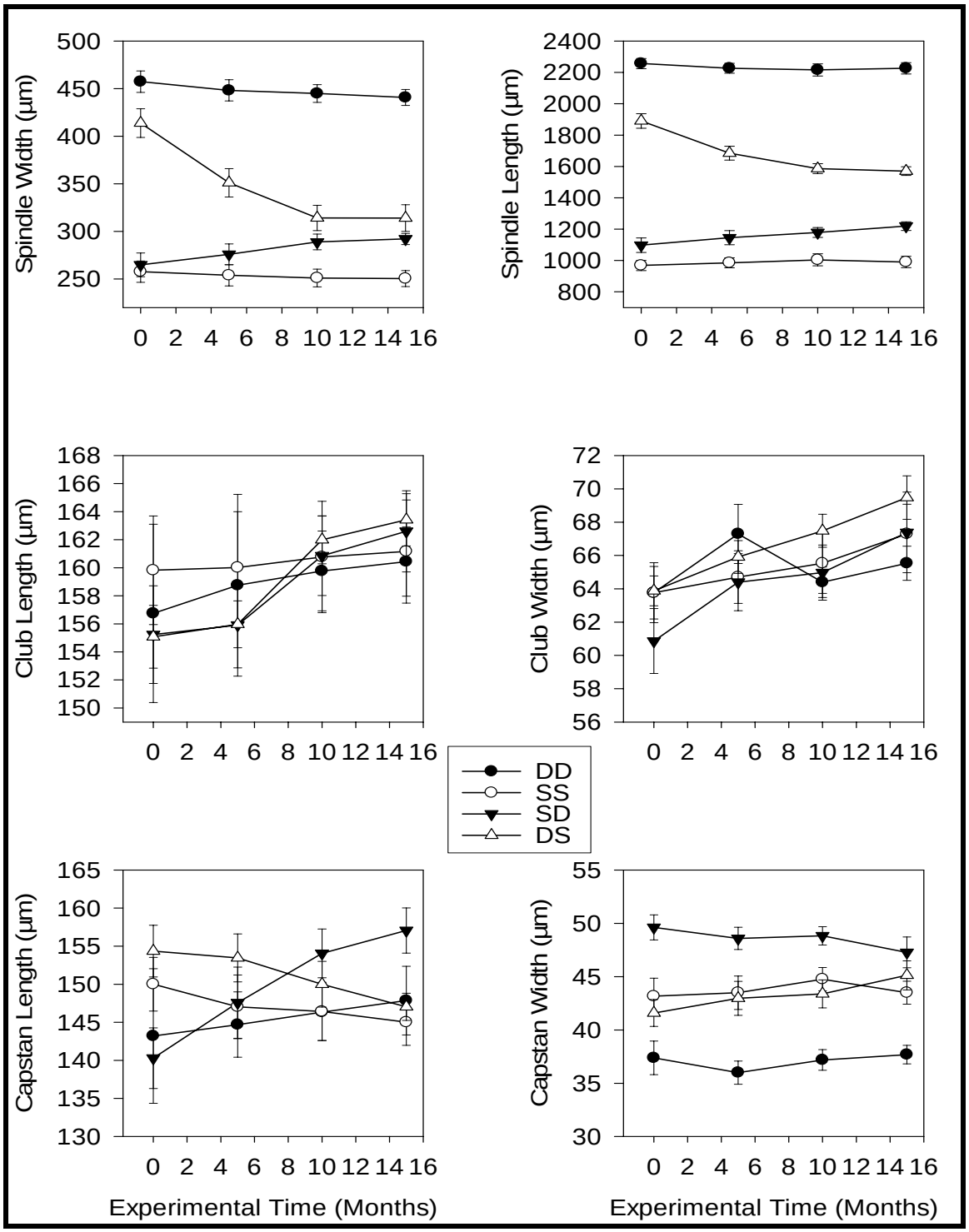

FIG. 13. Reaction norms of morphological traits through time in transplanted colonies. Spindle changes are more drastic than club and capstans. Population codes are SS: Shallow to shallow; DD: Deep to deep for residents and SD: Shallow to deep and DS: Deep to shallow for reciprocal transplants. Values are given as mean value ± 1 standard deviation.

Table 9. Temporal linear regressions of branches transplanted to opposite depths [Shallow to deep (SD) and deep to shallow (DS)] and residents [Shallow to shallow (SS) and deep to deep (DD)]. Values are given in μm ± standard error (n=15 for DD and SS, while n=30 for SD and DS).

Group	INTERCEPT	SLOPE	R^2	F	N	P- value
Spindle Length						
DD	2429.76 ± 92.05	-21.82 ± 42.61	0.1	0.26	55	0.6123
SS	1130.9 ± 55.37	3.07 ± 25.63	0.1	0.01	47	0.9053
SD	974.97 ± 47.07	67.1 ± 21.79	0.01	9.48	63	0.0043
DS	2324.69 ± 212.59	-203.96 ± 98.41	0.12	4.3	91	0.0466
Spindle Width						
DD	481.65 ± 35.33	-5.29 ± 4.87	0.1	0.53	55	0.6698
SS	249.44 ± 13.64	3.56 ± 6.26	0.1	0.32	47	0.6727
SD	215.65 ± 10.85	30.98 ± 5.02	0.45	18.11	63	<0.0001
DS	416.35 ± 25.2	-31.35 ± 11.67	0.1	7.22	91	0.0091
Capstan Length						
DD	138.95 ± 6.94	4.34 ± 2.54	0.05	2.94	55	0.0924
SS	152.7 ± 4.1	-1.73 ± 1.5	0.03	1.34	47	0.2535
SD	138.92 ± 4.23	5.24 ± 1.54	0.16	11.61	63	0.0012
DS	151.14 ± 3.23	0.37 ± 1.18	1.10E-03	0.1	91	0.7531
Capstan Width						
DD	37.13 ± 1.58	0.14 ± 0.58	1.10E-03	0.06	55	0.8059
SS	42.55 ± 1.52	0.97 ± 0.55	0.06	3.09	47	0.0856
SD	49.44 ± 1.54	-0.58 ± 0.56	0.02	4.98	63	0.0297
DS	40.5 ± 1.61	1.28 ± 0.59	0.05	4.73	91	0.0323
Club Length						
DD	158.42 ± 4.68	0.57 ± 1.71	2.10E-03	0.11	55	0.7386
SS	156.2 ± 4.33	1.18 ± 1.58	0.01	0.56	47	0.4587
SD	152.02 ± 3.92	2.63 ± 1.43	0.05	3.39	63	0.0704
DS	154.36 ± 2.38	2.65 ± 0.87	0.09	9.25	91	0.0031
Club Width						
DD	62.53 ± 1.5	-0.1 ± 0.55	6.40E-04	0.03	55	0.8535
SS	64.18 ± 2.09	0.2 ± 0.76	1.50E-03	0.07	47	0.796
SD	59.57 ± 2.65	2.16 ± 0.96	0.07	5.02	63	0.0286
DS	63.38 ± 1.4	1.48 ± 0.51	0.09	8.38	91	0.0048

Table 10. Repetitive measurements ANOVAs evaluating the effects of population (transplanted and residents), time (every 5 months until 15 months) and their interaction.

Source of Variation	DF	SS	MS	F	P
Spindle Length					
population	3	47065720	15688573	47.719	**<0.001**
time	3	179099.8	59699.93	1.898	0.132
population x time	9	2762789	306976.5	9.759	**<0.001**
Total	259	76277451	294507.5		
Spindle Width					
population	3	47065720	15688573	47.719	**<0.001**
time	3	179099.8	59699.93	1.898	0.132
population x time	9	2762789	306976.5	9.759	**<0.001**
Total	259	76277451	294507.5		
Club Length					
population	3	234.622	78.207	0.284	0.837
time	3	1794.632	598.211	6.281	**<0.001**
population x time	9	672.131	74.681	0.784	0.631
Total	259	37322.09	144.101		
Club Width					
population	3	815.748	271.916	2.872	**0.044**
time	3	346.814	115.605	5.339	**0.002**
population x time	9	336.717	37.413	1.728	0.085
Total	259	11367.29	43.889		
Capstan Length					
population	3	593.689	197.896	0.492	0.689
time	3	1558.5	519.5	3.198	**0.025**
population x time	9	4025.042	447.227	2.753	**0.005**
Total	259	60571.48	233.867		
Capstan Width					
population	3	3421.001	1140.334	14.618	**<0.001**
time	3	69.247	23.082	1.759	0.157
population x time	9	213.466	23.718	1.808	0.069
Total	259	10890.16	42.047		

A summary of the direction and magnitude of the spicular change after transplantation is presented in Table 11. The most salient feature is the substantially higher percentage (positive and negative) in the reciprocally transplanted colonies (DS and SD) compared to residents (SS and DD). SL and SW showed the highest percentage

variation for DS (-21.5 and -23%, respectively) and SD (5.9 and 7%, respectively). Two-way repetitive measurement ANOVAs (Table 10) showed significant differences among populations (DD, SS, SD and DS) for most traits (except CL and CAL) and time X population interaction in SL and SW.

Table 11. Percent variation of sclerite size of autotransplants (DD and SS) and reciprocal transplants (SD and DS) after 15 months. The values were obtained by acquiring the percent after subtracting the final and the initial values. Negative values represent the direction of the change (increase or decrease).

	SL	**SW**	**CL**	**CW**	**CAL**	**CAW**
DD	1.0	1.8	2.7	0.7	8.1	-0.8
SS	1.1	-2.2	1.1	1.5	-3.9	-0.6
SD	5.9	7.0	5.4	10.7	12.0	-3.2
DS	-21.5	-23.2	7.1	5.2	-8.6	8.5

3.3 *DNA Analysis*

To obtain the summary statistics of each group (e.g. habitat or reef) and to estimate the degree of connectivity among them, the data was divided into populations. The populations were first assigned by origin of habitat. All individual colonies found in either shallow or deep areas were classified as deep habitat or shallow habitat. Morphology was used to define the second group of populations (deep-type and shallow-type). The two classification schemes are not identical, as the atypical colonies found during the morphological analysis could be correctly classified. Also, each reef was treated as one population so that among reef comparisons could be established.

3.3.1. *Msh1*

A total of 130 sequences of the mitochondrial gene msh1 (723 bp) were used for the genetic analysis. Genetic diversity statistics are summarized in Table 12. Among the total number of individuals, 10 haplotypes were identified ranging from three to nine depending on the population. The numbers of segregating sites were similar between populations, 9 and 8 polymorphic sites were observed for colonies inhabiting shallow and deep environments, respectively. Most of the haplotypes were singletons; this mutation pattern was more common in colonies of shallow habitats. The pairwise F_{st} between

shallow-type and deep-type was 0.877, calculated with Tamura-Nei model of substitution in Arlequin. The corrected average pair-wise difference between the shallow- and the deep-type was 4.16925 (the uncorrected pair-wise difference 4.77506). Overall, the divergence of the two types was 0.567%. Fu's Fs test for the shallow- and the deep-type revealed a significant departure from equilibrium only for the shallow-type (-3.05038, P=0.037). The excess of rare mutations observed in the shallow-type is consistent with population expansion or purifying selection. The Raggedness index for the "shallow" type was not significant (0.16076; P>0.10).

Table 12. Summary of the genetic diversity and neutrality tests for msh1 and 18S.

Msh1	n	S	h	π	θ	k	Tajima's D	Fu's Fs
Deep habitat	62	8	7	0.0025	0.0024	1.7900	0.1325	-0.1066
Shallow habitat	68	9	8	0.0012	0.0026	0.8590	-1.4401	-0.6220
Shallow-type	74	5	6	0.0007	0.0014	0.4720	-1.2123	-1.2400
Deep-type	55	3	4	0.0010	0.0009	0.7350	0.2458	-0.2691
Total	130	11	10	0.0036	0.0028	2.6080	0.7305	-0.1962
Romero (shallow and deep)	33	7	6	0.0039	0.0024	2.7950	1.7936	1.6678
Media Luna (shallow and deep)	81	10	9	0.0036	0.0028	2.6140	0.7855	0.3406
Islands Culebra	16	2	3	0.0007	0.0008	0.5250	-0.3301	-0.5230
18s								
Deep habitat	59	6	7	0.0061	0.0051	1.5390	0.4735	-0.3310
Shallow habitat	84	7	11	0.0054	0.0072	1.3580	-0.6289	-3.6790
Shallow-type	88	5	9	0.0035	0.0055	0.8870	-0.8620	-3.7390
Deep-type	55	5	6	0.0014	0.0044	0.3520	-1.6119	-4.4450
Total	143	9	15	0.0088	0.0079	2.2040	0.2719	-3.1000
Romero (shallow and deep)	32	7	8	0.0093	0.0089	2.3450	0.1507	-0.6260
Media Luna (shallow and deep)	90	6	11	0.0090	0.0063	2.2680	1.0800	-1.0770
Islands Culebra	21	6	5	0.0042	0.0066	1.0570	-1.1500	-0.9090

The shallow-type consisted of 6 haplotypes and the deep-type of 4 haplotypes, among which the maximum distance was 6 substitutions. There were 2 and 3 segregating sites in the shallow- and deep-type, respectively. The two most common haplotypes of shallow- and deep-type were represented by 54 and 30 individuals, respectively. Nucleotide diversity indices (π, θ) varied among locations, the highest values were usually present in colonies found in deep areas (Table 12).

3.3.2. *18S*

A 251bp fragment of 18S was sequenced from 143 colonies of *P. flexuosa*. Most of the sequences (90) were from Media Luna reef (colonies used in the transplant experiment), while 32 sequences were from Romero and an additional 21 from Culebra. Among all sequences, 15 haplotypes were discernable with colonies in shallow habitats presenting the highest haplotype diversity (h= 11). Among reefs, Media Luna had the highest number of haplotypes (h= 11). Nucleotide diversity (π) and (θ) values were similar among populations (Table 12).

3.3.3. *Analysis of molecular variance (AMOVA)*

AMOVA tests showed significant differentiation among populations (Table 13 and 14), regardless whether the populations were divided per habitat (shallow or deep) or by morphotype (shallow-type and deep-type). Nonetheless, population differentiation was maximized when the latter assignment was used. A comparison among reefs (Media Luna vs Romero) yielded non-significant F_{st} values, suggesting an extensive gene flow among these two reefs. Also, a comparison of all shallow habitats (Romero shallow, Media Luna shallow and Culebra) suggested homogeneity among populations. The genetic homogeneity within La Parguera is not surprising because of the short distance between the reefs. Even when including colonies from Culebra, which is 100 km northeast from La Parguera, genetic homogeneity was still recovered. However, when comparisons of reefs lacking the deep habitats (e.g. in Culebra only shallow areas were sampled), F_{st} values were significant, suggesting a significant population structure. As inferred from the AMOVAs the haplotype network analysis showed similar patterns (Fig. 14). Two groups were extracted from the haplotype network analysis, the first consists of the vast majority of sequences that have the shallow morphotype and the second group contains most of the deep morphotype.

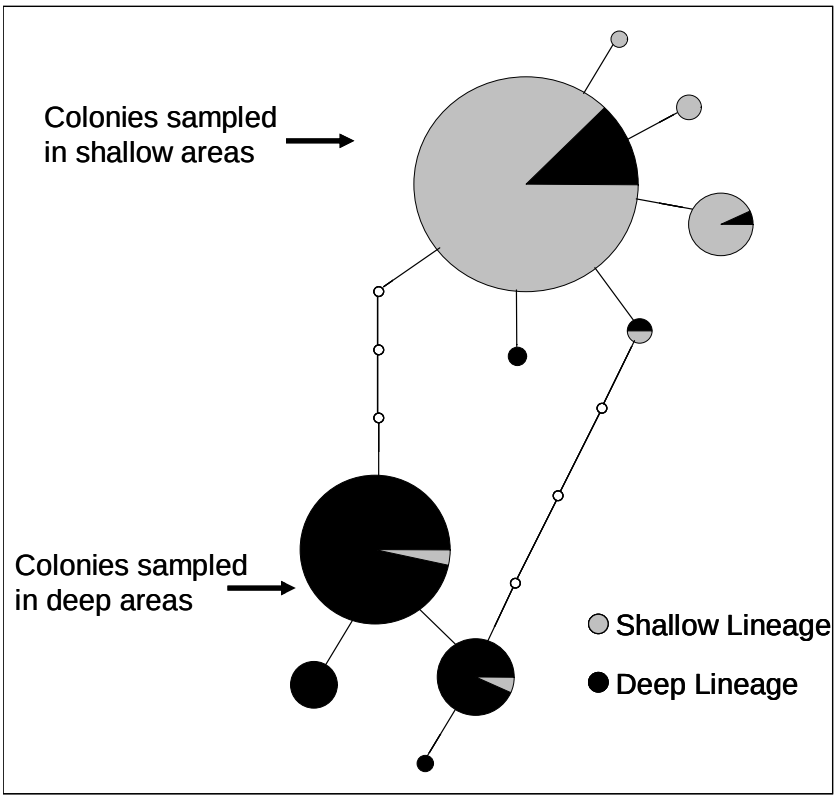

FIG. 14. Parsimony haplotype networks based on msh1 for colonies inhabiting shallow or deep areas. The network indicates the six haplotypes found in shallow areas (i.e. "shallow" type) and the four haplotypes found in the deep areas (i.e. "deep" type). The size of the circle is proportional to the observed number of sequences for the corresponding haplotype. The minimum number of steps is represented by the small empty circles.

3.3.4. *Gene genealogies*

Gene genealogies constructed in PAUP using Maximum Likelihood with the HKY and Jukes Cantor as most suitable substitution models for msh1 and 18S, respectively, as suggested by ModelTest. The HKY model was implemented with unequal base frequencies (A= 0.31140, C= 0.15640, G= 0.19090, T= 0.34130). Analysis performed using neighbor joining and parsimony yielded similar patterns. Also, the topology of msh and 18S was similar, however the 18S analysis recovered only one of the clades, as 18S is less variable than msh.

Table 13. Analysis of molecular variance (AMOVA) for msh1. Comparisons of population were made among depth profiles, morphotypes and reefs. The significance of the Φ_{st} values were obtained by randomizations of 10,000 permutations. $P < 0.001$ is represented by ***. Sampling in Culebra is limited to shallow habitats.

Comparison tested	d.f.	Sum of squares	Variance component	% variation	Φ_{st}
Between habitats (shallow Vs deep)					
Among populations	1	84. 89	1.29890 $_{Va}$	66.87	0.66873***
Within populations	128	82.36	0.64345 $_{Vb}$	33.13	
Total	129	167.254	1.94235		
Between morphotypes (shallow-type Vs deep-type)					
Among populations	1	130.412	2.06222 $_{Va}$	87.89	0.87891***
Within populations	128	36.084	0.28413 $_{Vb}$	12.11	
Total	129	167.254	1.94235		
Between reefs (Romero Vs Media Luna)					
Among populations	1	1.702	0.00829 $_{Va}$	0.64	0.00637
Within populations	128	165.552	1.29338 $_{Vb}$	99.36	
Total	129	167.254	1.94235		
Between reefs considering shallow populations only (Romero Vs Media Luna Vs Culebra, all shallow)					
Among populations	2	0.921	0.00157 $_{Va}$	0.37	0.00366
Within populations	65	27.844	0.42837 $_{Vb}$	99.63	
Total	129	167.254	1.94235		
Among reefs with different depth profiles (Media Luna Vs Romero Vs Culebra [no deep component])					
Among populations	2	15.034	0.18265 $_{Va}$	13.22	0.13223***
Within populations	127	152.22	1.19859 $_{Vb}$	86.78	
Total	129	167.254	1.94235		

Table 14. Analysis of molecular variance (AMOVA) for 18S. Comparisons of population were made among depth profiles, morphotypes and reefs. The significance of the Φ_{st} values were obtained by randomizations of 10,000 permutations. $P < 0.001$ is represented by ***. Sampling in Culebra is limited to shallow habitats.

Comparison tested	d.f.	Sum of squares	Variance component	% variation	Φ_{st}
Between habitats (shallow Vs deep)					
Among populations	1	55.599	0.79038 Va	49.27	0.49266***
Within populations	141	114.765	0.81394 $_{Vb}$	50.73	
Total	142	170.364	1.60431		
Between morphotypes (shallow-type Vs deep-type)					
Among populations	1	108.532	1.59683 Va	78.45	0.78455***
Within populations	141	61.832	0.43852 $_{Vb}$	21.55	
Total	142	170.364	2.03536		
Between reefs (Romero Vs Media Luna)					
Among populations	1	0.642	-0.01242 $_{Va}$	0.96	0.01043
Within populations	107	128.744	1.20321 $_{Vb}$	99.04	
Total	108	129.385	1.19079		
Between reefs considering shallow populations only (Romero Vs Media Luna Vs Culebra, all shallow)					
Among populations	2	1.790	0.00257 $_{Va}$	0.30	0.00305
Within populations	74	62.210	0.84067 $_{Vb}$	99.70	
Total	76	64.000	0.84324		
Among reefs with different depth profiles (Media Luna Vs Romero Vs Culebra [no deep component])					
Among populations	2	4.639	0.03526 $_{Va}$	2.91	0.02911***
Within populations	120	141.101	1.17584 $_{Vb}$	97.09	
Total	122	145.740	1.21110		

The genealogy divided the individuals in two clades (Fig. 15), which are highly indicative of the habitat origin (shallow or deep). In the bottom clade, 62 of the colonies are from deep areas; however nine colonies fell within this clade. These nine colonies are the atypical colonies found during the morphological analysis (e.g. Media Luna shallow), where comparisons of morphological characters of these atypical colonies did not match the overall population mean, despite living in the same habitat. Similar patterns are displayed for the top clade, which represents 68 colonies sampled in shallow areas, and three atypical colonies found in deep areas. Furthermore, these atypical colonies in each clade are those colonies that were misclassified by the discriminant function analysis. Table 15 shows a summary of the number of individuals within each genetic lineage divided by habitat and morphotype (shallow-type and deep-type). The shallow morphotype is more common in deep areas (9/62), while the deep-type (3/68) is less common in shallow areas. Furthermore, the atypical colonies within each habitat were later shown by the DNA analysis to be phylogenetically closer to their opposite habitat congeners, reinforcing the morphological differences found in univariate and multivariate analysis. Moreover, such colonies represent the misclassified (7%) colonies in the discriminant function analysis.

Table 15. Number of individuals in each genetic lineage. In the top the genetic lineages are divided by their living habitat. The bottom shows the genetic lineage division based on morphology (morpho-types).

	Habitat (shallow and deep)	
Genetic lineage	**Shallow**	**Deep**
G1	65	3
G2	9	53
	Morphotypes (shallow-type and deep-type)	
Genetic lineage	**Shallow-type**	**Deep-type**
G1	68	0
G2	0	62

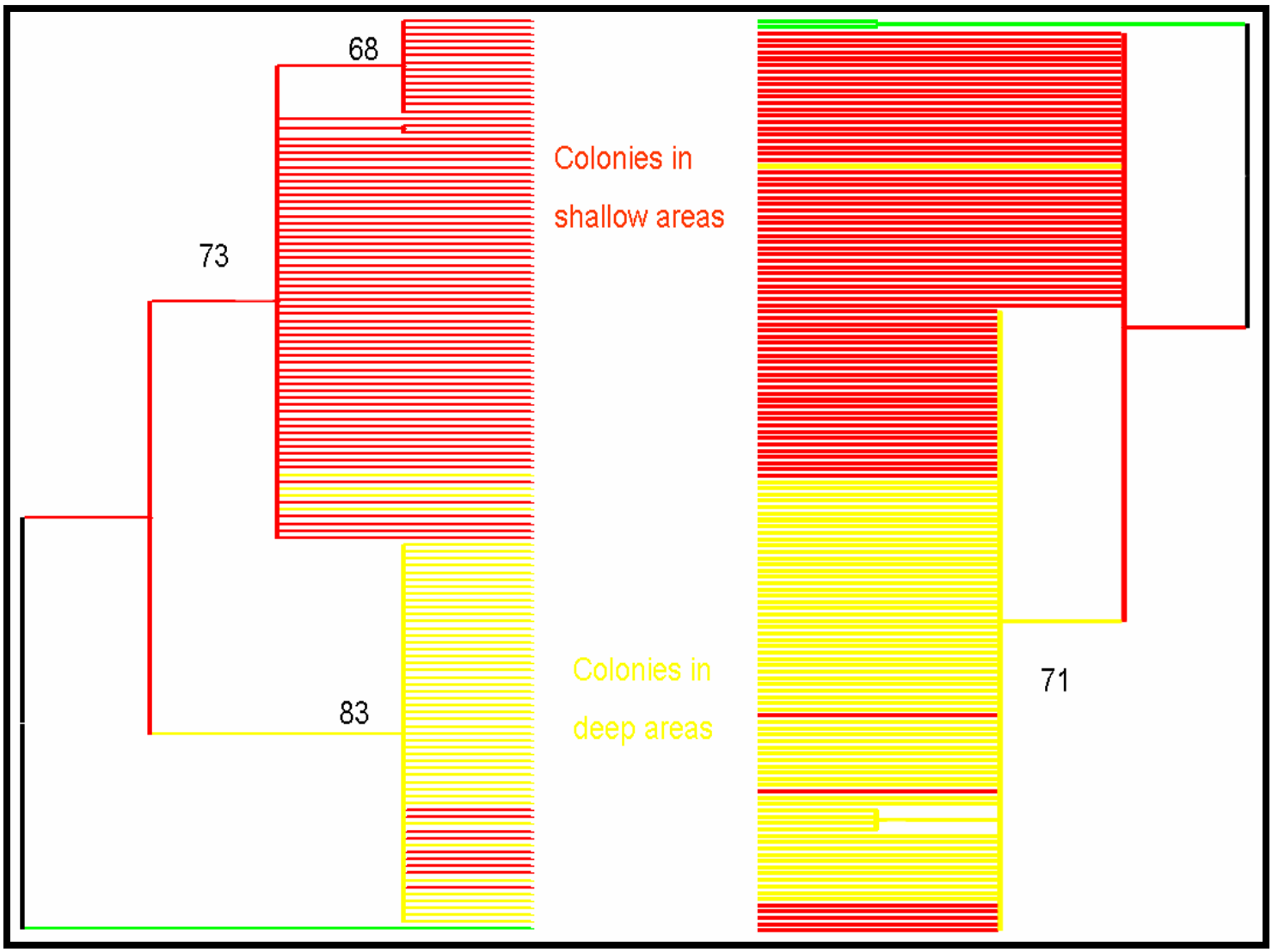

FIG. 15. Gene genealogies for msh1 (left) and 18S (right). Bootstrap values for 100 replicates in maximum likelihood (ML) are shown. Similar topologies and bootstrap values were recovered using maximum parsimony but not shown. *Muricea muricata* and *Eunicea* sp1 and sp2 were used to root msh1 and 18S genealogies, respectively. The colonies are divided by three colors. In red are colonies sampled in shallow areas, in yellow colonies in deep areas and in blue are the outgrops. The genealogy divided the individuals in two clades, which are highly indicative of the habitat origin (shallow or deep). In one clade (bottom of the left figure) most of the colonies (62) are from deep areas (yellow), however 9 colonies (represented in red) fall within this clade. These 9 colonies had a different morphology despite living in the same habitat. The same pattern was observed for the top clade.

4. DISCUSSION

The results suggest that *P. flexuosa* can be divided into two discrete morphological forms in southwest Puerto Rico. The first morphotype fits well with Bayer's description; adult colonies exhibit bushy-like shape and branch profusely in a single plane. Microscopically, size of leaf clubs, structural spindles and fused capstans are of ~200 μm, ~1000 μm and ~200 μm, respectively. This morphotype is pervasive in shallow areas (shallow-type), but a few colonies of the shallow-type can be found inhabiting deep habitats. The second morphotype (deep-type) could be described as small colonies, with fewer terminal branches, more sparsely polyps, thinner branches and bigger spindles (~2000 μm). The deep-type is largely confined to deep areas with low water motion patterns, high sediment transport and lower light levels. The deep-type was predominantly found at the muddy bed at the base of fore reefs of inside and mid reefs.

The discrete morphological distribution is in concordance with previous reports (Kim et al. 2004). Colonies inhabiting various depth profiles and water motion regimens show significant differences in morphology. According to Bayer (1961), length of clubs, capstans and spindles are about 200, 200 and 2000 μm, respectively or more, which is consistent with the results found for colonies inhabiting deep areas (except El Hoyo and Weinberg). Furthermore, spindles of up to 4000 μm in length were found in deep areas of inner reefs, which has not been reported in previous work (Bayer 1961; Kim et al. 2004). However, the original description of the species does not include the variation found in spindles at shallow environments, where the average spindle length was ~1000 μm.

Morphological differences of sclerite size within and among colonies inhabiting different depth profiles have been previously reported in octocorals. Spindles at the branch tip were significantly smaller and presumably underdeveloped in this study, contradict observations in studies of *Briarium asbestinum* (West 1997), reporting bigger spindles at the tips, presumably used as predator deterrent. The discrepancy of the findings may be related to different ecological pressures on the two species, different function of spindles in each species (i.e. *B. asbestinum* lacks central axis) or may depict difference responses constrained by their different phylogenetic history. Moreover in

terms of habitat differences, smaller spindles at high water motion areas are thought to increase stiffness of the colony to avoid breakage. Grigg (1972), West et al. (1993) and Kim et al. (2004) reported that sclerite size increased with decrease in water motion patterns for *Briareum asbestinum* and *P. flexuosa*. However, the opposite observation has been made in *Eunicella singularis* (Skoufas 2006). Colony size, calice diameter and polyp density may compensate water flow changes and enhance respiration, feeding or structure as has been reported for scleractinian corals (Bottjer 1980; Sebens and Done. 1992; Lesser et al. 1994; Bruno and Edmunds 1997; Sebens et al. 1997). Furthermore, the overall discrepancy in colony development (more and bigger terminal branches) may be interpreted as a response to the mechanical stimulation (drag forces) acting upon the colony, yet colonies in shallow areas (more developed) are exposed to higher water flow and vice versa.

In *P. flexuosa,* the differences of the two morphotypes are related to depth profiles, water motion and sediment transport. However, as water motion, sediment transport and light co-vary along depth profiles, the study could not distinguish the individual influences. Furthermore, the deep habitats of the inside and mid reefs differ significantly from those of the outer reefs. In the inside and mid reefs, loose sediments move along the slope and are deposited at the base of the forereef, where deep colonies were sampled. Therefore, settling larvae in such areas are subject to higher bed load (i.e. sediment movement) and must have developed an adaptational response to such environmental condition. Similarly, colonies in the two outer reefs, El Hoyo and Weinberg, developed in the edges of sand patches were different than those spread along the hard ground bottom. In La Parguera, the octocoral community observed at the sand bed adjacent to the forereef (~ 20m), is less diverse than the community of hard ground habitats. In such suboptimal habitats, *Pseupterogorgia* spp, *Plexaurella* spp. and some *Muricea* spp. (except *Muricia muricata*) dominate such areas. Therefore, these species are adapted to overcome sediment suffocation and develop. In the case of *Pseupterogorgia* spp. and *Muricea* spp. their lack of thick central axis allows for faster growth rates and rapidly reach a safe size from sediment burial. In *Plexaurella* spp., the presence of big polyps can efficiently remove sediment from the colony. The deep-type

of *P. flexuosa*, is a thin colony with a small central axis and few polyps, which might grow fast enough to bypass sediment overload.

The morphology of *P. flexuosa* can also be affected by light (e.g. availability of light for their symbionts) and other factors associated with depth (e.g. food resources, presence of predators and hydrostatic pressure). Nonetheless, water motion covaries with depth and therefore both are related. Light is likely a major factor related to depth and affects anthozoan morphology; as presence of zooxanthellae is tightly related to carbonate uptake and energy supply for the host. There is a number of studies showing the effect of variation in light levels on anthozoan morphology due to changes in zooxanthellae concentration and decrease of calcification rates (Graus and Macintyre 1976; Willis 1985; West et al. 1993; Meroz et al. 2002; Stambler and Dubinsky 2005). Moreover, plate-like colony shape is likely to be an evolutionary response to compensate for low levels of light by increasing area and zooxanthellae concentrations.

The two morphologies of *P. flexuosa* associated with different habitats showed some degree of phenotypic plasticity in sclerite characters (especially spindles), which showed a clear tendency to increase and decrease when transplanted to deep and shallow areas, respectively. Nonetheless, after the 15 months of the experiment in neither case the transplanted colonies became similar in size as the residents' spindles. The variation was higher for spindle length and width than for other traits. The results suggest that either, there was not enough time to allow the colonies to produce new tissue under the novel conditions; there are some morphological developmental constrains that restrict the plasticity of adult gorgonians (e.g. plasticity under larval stages) or there is a non environmental factor accounting for the rest of the morphological variation. There is less evidence for the former; colonies grew on average ~2 cm during the transplant experiment, and since the analysis was performed using tissue 1cm from the branch tip, there was more than 0.5 cm tissue to be analyzed. Also, the reaction norm graphs showed a stabilization of the curve at 10 and 15 months, suggesting that the maximum of variation was reached. However, other environmental factors such the 2005 Caribbean wide bleaching event, which was severe in Puerto Rico (Prada et al. unpublished data), may have affected the results. Alternatively, plasticity may be most likely promoted during the early life stages rather than in mature colonies.

The possibility that no environmental factors account for the majority of morphological variation is supported by the genetic data. Phylogenetic analysis of the msh1 and 18S genes showed two major lineages associated with depth. Mortality rates (other than by detachment or by overgrowth) of the transplanted colonies showed habitat preference for each lineage. Colonies transplanted from shallow to deep habitats suffered about 50% mortality; those transplanted from deep to shallow suffered 20% mortality by fall 2005. Since survivorship is a fundamental attribute of fitness then increase in mortality would indicate lower fitness. Therefore, each morphotype is better adapted to the deep or shallow areas. However, the results from the transplant experiments may represent experimental error or a drastic response of the colonies to extreme novel conditions. Regardless, the observed rates of mortality were higher than expected. Previous studies have shown that adult colonies (>20 cm) in natural populations are stable and have a normal survivorship rates above 90% (Yoshioka and Yoshioka 1991), suggesting that such high mortalities are natural responses rather than sampling error.

Regardless the two genetic lineages associated with the two habitats; there is a noticeable response of both shallow- and deep-type to environmental stimuli. As previous studies have reported, there is a positive correlation of spindles to increase in size as depth increases and vice versa (West et al. 1993; Kim et al. 2004). The transplantation experiment was not able to establish a clear trend in change for the other two sclerite types. Clubs and capstans slightly changed with depth but the tendency was not consistent and the change was small.

In Puerto Rico, the discrete morphological variation found in colonies of *P. flexuosa*, which clearly define two morphological groups with different habitats and their own phenotypic plasticity is genetically based. Also, it is likely that each morphotype is adapted to its own habitat. Mortality rates of reciprocal transplants are significantly higher than in natural populations, suggesting that the environment induces mortality in suboptimal areas. Gene genealogies, haplotype networks and AMOVA analysis of both nuclear and mitochondrial genes suggested that such discrete morphological distribution is correlated with the presence of two distinct lineages, distributed non-randomly in shallow and deep environments. However, either of the two lineages can infrequently be found in both depth habitats. The genetic break found in nuclear and mitochondrial DNA

is suggesting that gene flow ceased a long time ago and divergence may have led to speciation. Fixed differences in both nuclear and mitochondrial genes are comparable to those reported between species of octocorals (Sanchez et al. 2003; Wirshing et al. 2005; McFadden et al. 2006). Therefore, the current description *sensu* Bayer (1961) of *P. flexuosa* is a complex of at least two distinct genetic lineages, adapted to different habitats and that do not exchange genetic material despite living in sympatry. The extensive distribution and ample morphological variation corresponds to two distinct genetic lineages with narrower distributions and more rigid phenotypic plasticity. The observed genetic pattern may have resulted from 1) secondary contact after populations diverged in allopatry and reproductive incompatibility developed, 2) by divergence with gene flow through ecological specialization in sympatry or 3) by the poorly understood process of hybridization in anthozoan evolution.

4.1. *The speciation process*

Anthozoans as other marine invertebrates can theoretically disperse over long distances due to the lack of obvious physical barriers. Furthermore, it has been suggested from laboratory and field experiments that larvae of most broadcasters such as *P. flexuosa* are capable of staying days to weeks in the water column and not surprisingly, to produce genetically homogeneous populations in larger geographic areas (Hellberg 1996; McFadden et al. 1997; Lessios et al. 1998; Lessios and Robertson 2006). Despite the high potential for dispersion and genetic connectivity, allopatric speciation is probably the most common mode of speciation in marine environments (Mayr 1942; Knowlton and Mills 1992; McCartney et al. 2000; Lessios et al. 2001; Barber et al. 2002; Avise 2004; Fukami et al. 2004a). Furthermore, recent genetic studies (Gutiérrez-Rodríguez and Lasker 2004; Baums et al. 2005; Taylor and Hellberg 2005; Vollmer and Palumbi 2007) have contradicted earlier assumptions of population homogeneity in Caribbean populations of marine taxa (Briggs 1973; Shulman and Bermingham 1995). Even though allopatric distribution could have caused the divergence of the lineages, there is not recent evidence of geological processes that may have altered the patterns of Caribbean circulation. However, distinct lineages have been uncovered in other Caribbean invertebrates (Torres-Pratts pers. comm.; Zardus and Hadfield 2005).

On the other hand, sympatric speciation by ecological differentiation (Doebeli and Dieckmann 2000) and disruption of gene flow in proximate populations is plausible. The two genetic lineages found in this study are found at the opposite ends of a depth gradient. So it is possible that diversifying selection may favor the two phenotypes at the extremes of the depth gradient, preventing gene flow through assortative mating and eventually leading to new species (Palumbi 1992). These ecological specializations to depth habitats have been pointed out in earlier reviews (Knowlton and Jackson 1994). Furthermore, such ecological differences in habitat distribution or niche utilization may be reinforced by dissimilar characteristics associated with the habitats they occupy. Symbiotic relationships to host (Diekmann et al. 2003; Santos et al. 2004) or to environment (Rowan and Knowlton 1995), differential timing of gamete release during spawning events due to depth related differences (Lessios 1984; Knowlton et al. 1997; Szmant et al. 1997) may have provided different resources to populations at different habitats and eventually prevent organisms to reproduce randomly. As a consequence, rapid evolution of mating systems may have been favored (Palumbi and Metz 1991; Palumbi 1994; Metz and Palumbi 1996; Vacquier et al. 1997; Palumbi 1998; Hellberg and Vacquier 1999; Riginos and McDonald 2003; Mah et al. 2005) In Caribbean corals, diversifying selection has been reported in at least two species; in *Montastraea annularis* (Weil and Knowlton 1994) and *Favia fragum* (Carlon and Budd 2002). It is likely that the genetic differences reported by Brazeau and Harvell (1994) in the common gorgonian *B. asbestinum* could have arisen through the same mechanism. Although, disruptive selection seems to explain the divergence of the two lineages of *P. flexuosa,* hybridization is also another plausible mechanism to cause diversification in marine taxa.

Hybridization is a common phenomenon in plants and the rise of new lineages due to reticulations is often reported in flowering plants (Rieseberg et al. 1990; Rieseberg 1991; Arnold 1997). In animals, including marine taxa hybridization is not uncommon. There are instances of hybridization in angelfishes (Pyle and Randall 1994), African cichlids (Schelly et al. 2006), cyprinid fishes (DeMarais et al. 1992; Gerber et al. 2001), butterflies (Scriber and Ording 2005; Gompert et al. 2006; Mavárez et al. 2006), suggesting that hybridization is an important evolutionary mechanism for speciation in animals. The phenomenon of reticulate evolution may have great influence in the

evolution and diversification of marine species especially those living in sympatry and with high potential for hybridization (e.g. spawners). Chromosome incompatibilities or tight gene connections due to rearrangements generated by hybridization can prevent backcrosses, stimulating speciation.

Veron (1995) has provided a theoretical framework to consider reticulate evolution as an important factor of coral evolution. Direct measures of chromosome differences established in *Acropora* (Kenyon 1997), genetic surveys of the nuclear genome of corals (van Oppen et al. 2000; Vollmer and Palumbi 2002; Miller and van Oppen 2003; Willis et al. 2006) and direct crosses of gametes (Hatta et al. 1999) have shown introgression in natural populations. In *P. flexuosa,* the two uncovered lineages may have arisen by hybridization between the common form of *P. flexuosa* with another *Plexaura* or *Eunicea* species. Yet, it is uncertain whether *P. flexuosa* is phylogenetically closer to *Eunicea* or *Plexaura* (Sanchez et al. 2003; Sanchez and Wirshing 2005; Wirshing et al. 2005). Furthermore, hybrid fitness may increase over parent fitness in novel environment or in extreme habitats. Hybrids tend to explore novel habitats to avoid introgression and competition with their parents (Dowling and Secor 1997; Burke and Rieseberg 2003; Gompert et al. 2006). Therefore, it is likely that the lineage related to deep muddy areas is possibly of hybrid origin and is better adapted than its parent species to such conditions.

5. CONCLUSIONS

Morphological and genetic data suggest the presence of two discrete lineages in *Plexaura flexuosa*. The two types are associated with different depth profiles, however, both can be found in either depth, yet not randomly. Water motion and the effect of sediment transport during recruitment may be associated with the non-random distribution. Therefore, the *P. flexuosa*'s extensive distribution and ample morphological variation corresponds to two distinct genetic lineages with narrower distributions and more rigid phenotypic plasticity than the original description. The accepted description *sensu* Bayer (1961) of *P. flexuosa* is a complex of at least two distinct genetic lineages, adapted to different habitats and that do not exchange genetic material despite living in sympatry. The present study highlights the importance of correctly defining species, because the unknowingly use of species complexes can overestimate geographical distribution, population abundance, and physiological tolerance. Consequently, decisions based on these estimates will have repercussions in conservation programs (Knowlton and Jackson 1994). Furthermore, the non-random distribution of both morphotypes can yield misleading results in the absence of adequate taxonomical distinctiveness.

The observed genetic patterns may have resulted from ecological specialization of the two forms in sympatry or by the poorly understood process of hybridization in anthozoan evolution. Studies of the mechanism by which anthozoans achieve assortative mating and become reproductively isolated would give us insights in the speciation process. Also, cross fertilization experiments, genetic assessment of shared alleles through genetic markers and karyotyping may shed light on the speciation process via hybridization. Reticulations are common in plants, a group that resembles most of the ecological aspects (bet hedging strategies, modular organization, philopatric recruitment, etc.) that govern marine modular organisms.

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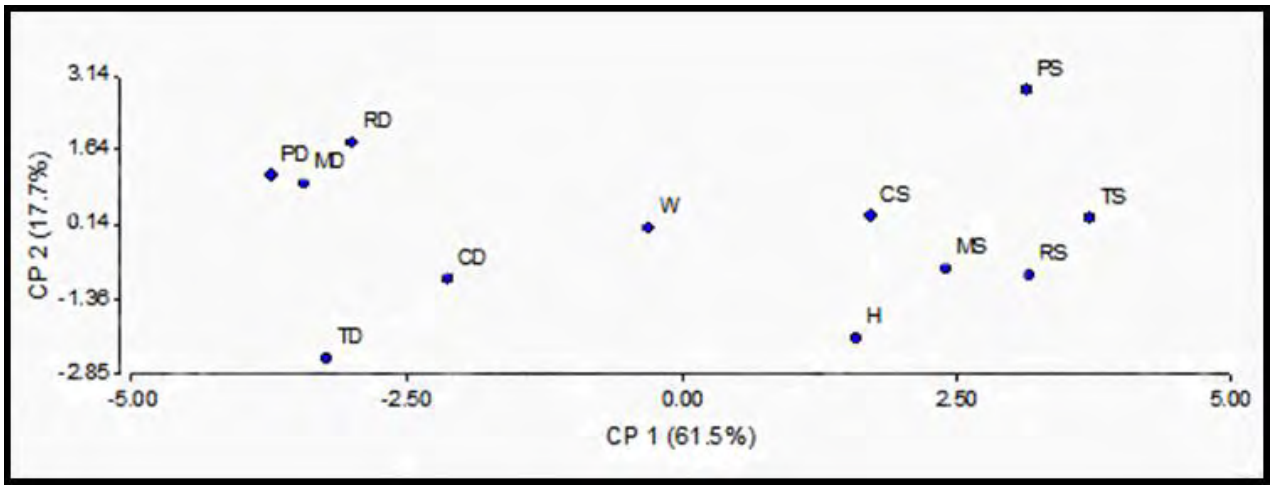
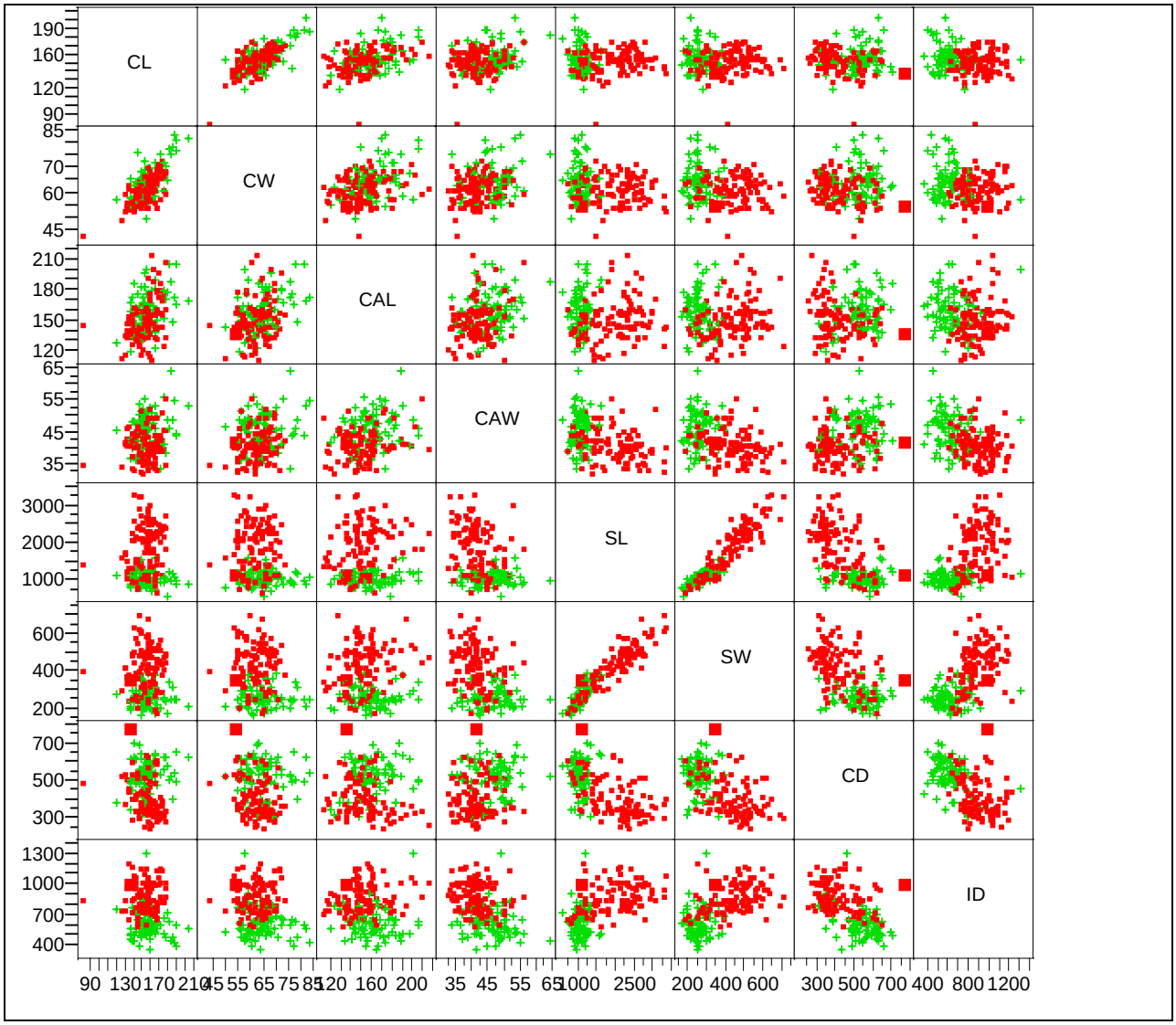
APPENDICES

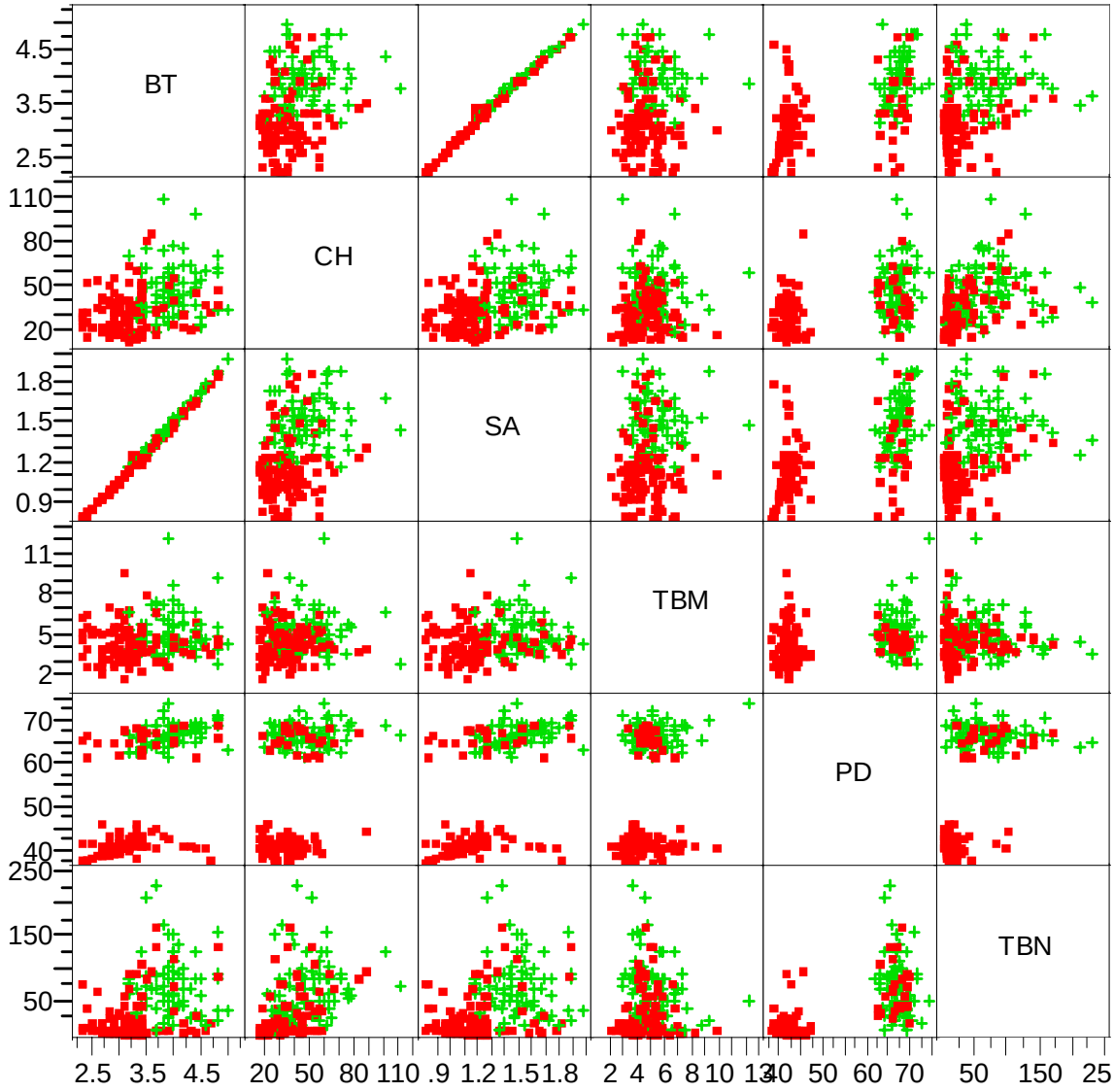
Appendix 1. Principal component analysis on transformed data (0,1 scale) of 14 phenotypic traits in 12 locations of *P. flexuosa.* Axes are the first two principal components. Location codes are as in Table 1.Note that analysis on raw data yielded similar results.



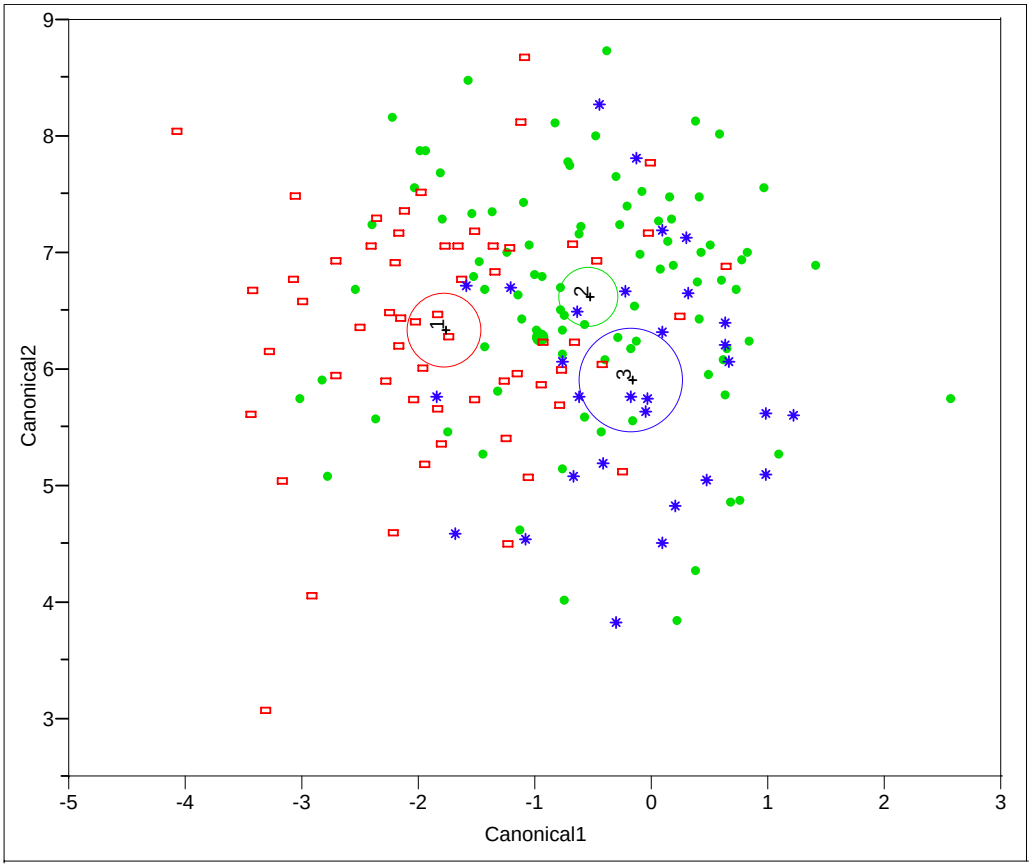
Appendix 2. Multivariate scatterplot matrix of micromorphological features. Codes are as in table 1. Colonies in Green crosses and red dots are for colonies in shallow and deep areas, respectively.



Appendix 3. Multivariate scatterplot matrix of micromorphological features. Codes are as in table 1. Colonies in Green crosses and red dots are for colonies in shallow and deep areas, respectively.



Appendix 4. Discriminant function analysis plot based on 12 morphological characters of *P. flexuosa* colonies inhabiting different zones (1 for inner, 2 for mid and 3 for exposed). Multivariate comparison (fixed-effect MANOVA) among depths was significant (Wilks' = 0.6598688; F = 3.1960; df = 24/332; P < 0.001). Misclassified colonies = 75 (42%).



Appendix 5. Discriminant function analysis plot based on 12 morphological characters of *P. flexuosa* colonies inhabiting different locations (PD = Pelotas deep, PS = Pelotas shallow, CD Conservas deep, CS = Conservas shallow, RD = Romero deep, RS = Romero shallow, TD = Turrumote deep, TS = Turrumote shallow, MD = Media Luna deep, MS = Media Luna shallow, H = El Hoyo and W = Weinberg). Multivariate comparison (fixed-effect MANOVA) among depths was significant (Wilks' = 0.0193656; F = 6.1063; df = 132/1302; P < 0.001). Misclassified colonies = 70 (39%).