MOLECULAR RELATIONSHIPS IN POTENTIALLY HYBRIDISING SPECIES OF THE *ACROPORA 'PALMATA'*-GROUP

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Thesis
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September 1999
Biochemistry and Molecular biology / Mariene biologie
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The picture on the front page shows two coral colonies from a reef near Bonaire. In the back a coral colony of *Acropora palmata* and in the front a coral colony of *A. prolifera* can be seen.

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Summary

Molecular relationships were investigated among the three Caribbean *Acropora* species *Acropora palmata*, *A. cervicornis* and *A. prolifera*. To examen the possibility of interspecific hybridisation occurring in the field sequence analysis of rDNA ITS1, ITS2 and the 5.8S coding region and Pax-C intron was performed.
Phylogenetic analysis showed no or hardly any genetic structure and uncorrected p-distance showed a maximum of 9.84 % sequence difference for the total region (ITS1 and 2, and 5.8S). In addition, variances calculated with Analysis of Molecular Variance showed no significant fraction of the total genetic variance being partitioned among species. These results may be correlated with interspecific hybridisation events – whether occurring at present or in the past. Results from other research projects involving morphological features, morphometrics and reproductive characters (Stockwell and Willis pers comm), spawning time (Szmant 1986) and fossil record (Budd et al. 1994) of these coral species, support this. However, the three Caribbean *Acropora* morphospecies could also have descended from a common ancestor and the sharing of similar sequence repeats may represent ancestral polymorphism. Whether speciation is occurring and the three species are diverging or merging is not known and questions concerning the mechanism behind the maintenance of different morphological features remain unclear.

Introduction

Coral diversity on the reef today is threatened by several influences including storms (Jones and Endean 1973; Rützler and MacIntyre 1982) and climate changes – resulting among others in coral bleaching – and human impact as the result of urbanisation, tourism and coastal development (Ogden et al. 1994; Meesters et al. 1994; Fiege and Neumann 1994; Zann 1994; Bak and Nieuwland 1995). Changes in coral cover over time and a decrease in numbers, during the last two decades could be observed (Bak and Nieuwland 1995; Meesters et al. 1994). To maintain this precious underwater habitat, conservation and maintenance is required. To make important decisions on this level, it is necessary to obtain knowledge concerning factors including population structure, evolution, ecology, growth and reproduction.

Systematics and morphology of corals

When studying corals, one of the very basic items is systematics because basic knowledge of which species is involved is important for performing research on other levels. In systematic research, an attempt is made to define or redefine taxa by a combination of all available information from biological, molecular and other relevant areas of science. In the past, for corals this has merely been done based on morphological features (Veron 1995; Miller and Babcock 1997). However, a clear classification of species could not be made in all cases.

Corals are known to have a high morphological plasticity due to environmental and genetic variation (Foster 1979; Willis and Ayre 1985; Van Veghel and Bak 1993; Veron 1995). This makes determination of species boundaries difficult (Gattuso 1991; Veron 1994; Wallace and Willis 1994).

An example of phenotypic plasticity and taxonomic difficulties in corals can be found in *Montastrea annularis*. Three different morphotypes exist, which are 'bumpy', 'columnar' and 'massive' and even intermediate morphothypes are present on the reef – although these are only infrequently observed (Foster 1979). In *Montastrea annularis* and *Siderastrea siderea*, several mature colonies have been transplanted between different reef environments (shallow and deep) in Jamaica. In a study on skeletal morphologies, corallite characters were analysed using multivariate analysis of colonies from different depths, a range in light intensity, water activity, sedimentation rate and food availability. Results showed that both coral species displayed plastic response of phenotype to the environment (Foster 1979). However, life history aspects apart from growth appeared to be significantly different among the *M. annularis* morphotypes and independent techniques (i.e. growth rate, banding and isotopic comparisons) provided completely consistent confirmation of the specific distinctiveness of the three shallow water colonies (Knowlton et al. 1992; van Veghel and Bak 1993). Although no difference in spawning time and behaviour could be observed (van Veghel 1994) significant differences in fecundity and other reproductive characteristics could be found between the three morphotypes (van Veghel and Kahmann 1994). However, new data from studies on ITS sequences of nuclear ribosomal RNA (ITS1, 5.8S and ITS2), showed that the three proposed species (*M. annularis*, *M. faveolata*, and *M. franksi*) from Florida reefs to be a single evolutionary entity (Medina et al. 1999).

It is still not known which mechanisms influences morphology and on which level (genetic or environmental) this is determined in corals but it is obvious that applying systematics to corals is a complicated issue. Some phenotypic indicators of genotype variability can be observed directly. For instance, growth form changes in response to transplantation provide a clear indication of physical environmental influence on genotype expression. Experiments on *Turbinaria mesenterina* involving transplantation experiments show that 'flat' and 'convulted' ecomorphs have a phenotypic response to a depth-related factor, which is believed to be light. Similar research, which has been performed on *Pavona cactus*, including some genetic analysis, showed that specific morphotypes are associated with specific genotypes (Wallace and Willis 1994).
In *Stylopora*, the two growth forms showed different depth distributions and physiological characters. Compared with deeper living colonies, *S. mordax*, living at 1 m showed large differences in physiological and morphological characters. The rather massive growth form and the lower growth rate in the colonies growing at 1 m depth could be explained by water motion and light. This, however, could not account for some of the differences observed (Gattuso et al. 1991; Knowlton 1994) and it was decided that the '*mordax*' and '*pistillata*' ecomorphs of *S. pistillata* should be recognized again as separate species (Gattuso et al. 1991; Wallace and Willis 1994).

Coral taxonomy and involved species concepts

In coral taxonomy describing and naming taxa is done according to the rules of nomenclature, while at the same time an attempt is made to meet the requirements of a currently acceptable species concept (Veron 1995; Wallace and Willis 1994). Linnaeus started to define the diversity of life in the last century using a system in which he hierarchically classified species based on morphology. A species was given two names consisting of a genus and a species name. Later, species were classified based on common ancestors. Species were believed to evolve gradually from common ancestors and could be classified in a hypothetical phylogenetic tree. Therefore, species in the same genus were more similar because they originated from a more recent common ancestor than species from different genera (Futuyma 1998).
However, it is not easy to define a species or a species concept that can be applied in general to classify the diversity of life. Several attempts have been made and today five major species concepts exist, but they all seem to have little bearing on operational coral taxonomy (Veron 1995). One of the concepts involves biological species (Mayr 1942; Veron 1995; Futuyma 1998). In this concept biological species are seen as units within which genes are, or can be, freely exchanged, but between which gene flow does not occur (at least under normal circumstances). Consequently, species were considered as reproductively isolated from other species. In the evolutionary species concept, species are based upon developmental, genetic and ecological constraints, not just heredity. Species are seen as populations that have had a common evolutionary history. Besides the biological and evolutionary species concept there are three others which are the recognition, the cohesion and the phylogenetic species concept. They are respectively based on the most inclusive population of biparental organisms which share a common fertilisation system, having the potential for cohesion through intrinsic cohesion mechanisms or having a unique combination of characters based on which the species can be recognised (Avise 1994; Futuyma 1998). Today, in coral taxonomy the biological species concept is still used, assuming morphological differences between

coral species to be correlated with reproductive isolation (Wallace and Willis 1994; Miller and Babcock 1997).

Reproduction in corals

In corals both asexual and sexual reproduction forms can be found. Asexual reproduction can take place in the form of fragmentation or asexual production of larvae. Sexual reproduction can occur during the release of gametes into the water after which fertilisation and development of planula larvae occurs. Another form is the brooding of planula larvae after internal fertilisation. When the cycle is completed the larvae will be released into the water after which settlement will occur (Harrison et al. 1984; Veron 1995). Broadcasting of gametes for external fertilisation during a brief annual spawning is now seen as probably the most common mode of reproduction among scleractinians (Harrison et al. 1984; Veron 1995; Willis et al. 1997).

For the majority of the broadcasting species on the Great Barrier Reef, Australia, spawning has been shown to occur on the same nights. In addition, the time of spawning (hours after sunset) was generally consistent within each population and between populations at different sites. In 17 of the 33 Great Barrier Reef species that were studied at more than one reef, allopatric populations spawned within an hour of each other on the same lunar day (Babcock et al. 1986). The eggs and egg-sperm bundles of most gamete-spawning corals, whether hermaphroditic or dioecious, are buoyant and float to the surface layers of the sea. After the egg-sperm bundles reach the surface they break apart, releasing the eggs and sperm. No sign of fertilisation were observed prior to the fragmentation of the egg-sperm bundles and the first signs of fertilisation were not observed until approximately 2.5 hours after spawning. Larvae did not become strongly mobile until approximately 36 hours after fertilisation (Babcock et al. 1986). However, more recent experimental breeding trials in *Platygyra* suggested that fertilisation occurred immediately after eggs had been introduced into the vials, independent of morphotype of the parental colonies (Miller and Babcock 1997).

Hybridisation, polyploidy and reticulate evolution

With this synchronised multi-species mass spawning in corals, gametes of different species become mixed and hybridisation may occur. Results from *in vitro* crosses between 42 species pairs from *Acropora*, *Montipora* and *Platygyra* showed that more than one-third of the pairs is capable of interspecific hybridisation (Willis et al. 1997). Coral species belonging to the genus *Acropora* are known to reach reproductive maturity after at least four years and to date *Acropora* hybrids have been maintained for up a few years only (Willis pers comm). Hence, nothing is yet known about fertility and other aspects of hybrids in this genus and more research has still to be performed (Willis pers comm; Wallace and Willis 1994). Hybridisation has been seen as to increase morphological variation within interbreeding units (Arnold 1997; Dowling and Secor 1997). This could be an explanation for the high morphological variation of scleractinian corals (Willis et al. 1997).

Hybridisation has already been studied in a number of plants and animals, although it was believed earlier that hybridisation in animals is rare (Arnold 1997; Dowling and

Secor 1997). This is probably due to the fact that untill recent times, less research on interspecific hybridisation has been performed in animals (Dowling and Secor 1997). In addition, the proportion of successful progeny from hybrids is much lower for animals, which was thought to be due to low levels of introgression (Dowling and Secor 1997). To date more studies have been done on animals including whales, fishes, insects, amphibians and reptiles (Dowling and Secor 1997; Dowling and Hoeh 1991; Bérubé and Aquilar 1998).
Veron (1995) has suggested that corals are like plants. A shared number of attributes including benthic origin, asexual propagation through fragmentation or fission, high fecundity and dispersal capacities, and polyploidy (Kenyon 1997) can be found in both plants and corals. In addition, the frequently found interspecific hybridisation in plants is one of the parallels with corals that has interested coral biologists. As a consequence of hybridisation, introgression and sometimes polyploidy can occur. This latter is an important mechanism of speciation, which can be found in both plants and animals (Kenyon 1997; Dowling and Secor 1997; Arnold 1997). Polyploidy is a chromosomal alteration in which an organism possesses more than two complete chromosome sets. In a karyotyping study of a range of *Acropora* species, polyploidy has been found in six instances (Kenyon 1997). Two categories of polyploidy exist namely allopolyploidy and autopolyploidy. Autopolyploid organisms derive a replicate chromosome set from a single parent species. In allopolyploidy, hybridisation between two chromosomally different taxa provides a hybrid, which is usually sterile. The hybrid species may be able to propagate itself asexually and fertility can sometimes be restored (Kenyon 1997). Polyploidy is thought to allow for adaptation to a wider range of environments provided by multiple sets of genes obtained (Dowling and Secor 1997). The result of introgression in hybridising species could have an impact on evolution in increasing the level of taxonomic variation and with this allowing evolution to proceed. New variations in species could inhabit niches, which have become available by changes of environment (Dowling and Secor 1997).

Reticulate evolution, coupled with hybridisation events, is dominated by sequential division and merging (Veron 1995) of clades and gene flow between different species (i.e. introgression). For corals it has been hypothesised that this may be based on surface circulation vicariance, causing taxa to become repeatedly isolated and reconnected (Veron 1995). Reticulate evolution, based upon chromosome numbers, has recently been proposed, to occur within the coral genus *Acropora* (Veron 1995; Kenyon 1997; Willis et al 1998; van Oppen et al., submitted) and *Platygyra* (Miller and Babcock 1997). In *Platygyra*, morphospecies are widespread throughout the Indo-Pacific and a varied level of differentiation and merging between the morphological or taxonomic units can be seen. Surface circulation vicariance mechanisms and reticulate evolution may well be the basis for the morphological and genetic variation in *Platygyra* populations across both local and geographic scales (Miller and Babcock 1997). In order to study reticulate evolution and hybridisation and speciation events proceeding from this the use of a large and extant coral genus would be ideal (Wallace and Willis 1994).

The coral genus *Acropora*

A coral genus, which is large and extant, is *Acropora*. With over 370 nominal species and around 150 valid species even after extensive revision, it is by far the largest extant reef-building coral genus. *Acropora* is widespread throughout the tropical Indian,

Pacific, and West Atlantic Oceans, where colonies are typically a dominant component of the shallow reef assemblage. In the geological record, the genus first appears in the Eocene and is widely distributed by the Miocene (Veron 1995). Records today, give evidence for up to 70 species of *Acropora* living sympatrically not being unusual (Veron 1993). All species within the subgenus *Acropora* are known to be hermaphroditic broadcast spawners. They release buoyant bundles of eggs and sperm that break apart at the surface of the sea, after which fertilisation takes place (Willis et al. 1985; Babcock et al, 1986). Many species spawn within one or two hours of each other (Babcock et al. 1986) and, because eggs and sperm of *Acropora* are viable for up to eight hours after release (Willis et al. 1997), this creates widespread opportunities for interspecific hybridisation and introgression and makes the coral genus *Acropora* an ideal subject for study of the nature and evolution of scleractinian reef coral species (Wallace and Willis 1994).

The Caribbean *Acropora* '*palmata*' group

In the Caribbean only three different morphospecies of the genus *Acropora* (Gregory 1895; Vaughan 1901; Vaughan 1919) can be found. Early in the 18th century Linnaeus described the genus in the Caribbean for the first time under the name *Millepora*. From that time it changed into *Madrepora* (Lamarck 1816) and later into *Isopora* (Vaughan 1901). At the end of the 19th century it was first suggested by Brook (1853) that the three species were probably only one. After a thorough examination, this one species complex was again devided into three distinct species in 1899, which was also supported by Vaughan (1901 and 1919). The three morphotypes are currently separated into three species *Acropora palmata*, *A. cervicornis* and *A. prolifera* (Vaughan 1919). He (Vaughan 1919) also mentioned forma cervicornis standing on one side and forma palmata on the other. Nothing however was mentioned about hybridisation and evolution.

Together with morphology habitat differs as well, although there can be some overlap. All three forms can be found throughout the whole Caribbean, the Florida keys, the Bahamas and the West Indies (Goreau 1959; Adey 1977; Rützler and Macintyre 1982; Budd 1994) and although broad zonation patterns divide the three species into different zones (Adey 1977; Goreau 1959), all three show overlapping patterns (Rützler and MacIntyre 1982). *Acropora palmata* has broad, flat, frond-like branches, forming colonies meters in diameter and is very common in turbulent shallow waters (1-8 m) (Figure 1A). *Acropora cervicornis* (Figure 1B) has more cylindrical branches, can form colonies up to 3 m high and can be found more on the outer ridge of the

R. Hays Cummins (http://jrscience.wcp.muohio.edu/TropEcolma

Figure 1A. *Acropora palmata*

Figure 1 B. *Acropora cervicornis*

outer fore reef deeper (till 24 m). *Acropora prolifera* (Figure 1C) has more crowded branches than *A. cervicornis* that are often crossing over and fusing, producing flabelliform or reticulate branches. Usually *A. prolifera* is smaller than *A. cervicornis* and not found in dense thickets. *A. prolifera* can be found on seaward side of reef crest (0.5-2 m) in very turbulent waters (Goreau 1959; Adey 1977; Rützler and MacIntyre 1981). According to Rützler and MacIntyre (1982), all three species are differentiated by growth form with *A. prolifera* being the intermediate, linking *A. palmata* with *A. cervicornis*.

All three Caribbean *Acropora* species are known to spawn at the same time in August and there is only one reproduction cycle per year (Szmant 1986). Evidence for the possibility of hybridisation in the form of *in vitro* hybridisation experiments (Willis pers comm) showed in crosses between *A. palmata* sperm and *A. cervicornis* eggs up to approximately 80-90% fertilisation and survival of viable hybrids for up to several days. This does not however, imply that hybridisation occurs in the field. Isolating mechanisms can operate on several levels of which two important types are premating and postmating. The first prevents the crossing of two different species and the second reduces the full success of the inter-specific cross (Veron 1995). Examples of the first are seasonal and habitat isolation or behaviour isolation and the latter, a reduced viability of the F1 hybrid or full viability but being sterile and being unable to reproduce sexually.

Figure 1C. *Acropora prolifera*

This morphology and habitat difference together with the uncertain taxonomic status of these three species, serves to be an interesting topic for new information on the role of hybridisation in speciation and evolution. In other organisms, hybrids were initially recognised as being intermediate between the two parent species although now it has been discovered that this does not necessarily have to be the case (Dowling and Secor 1997). Hybrid lineages have been identified by looking at morphological intermediates,

increased heterozygosity of nuclear gene loci and polyploidy of the species. In addition mtDNA variation has been investigated (Dowling and Secor 1997). Here I have applied molecular techniques to gain more information on whether natural hybridisation is occurring in the Caribbean *Acropora* '*palmata*' group and to unravel the evolutionary history of these morphospecies.

Nucleotide sequence comparison

Today with the discovery of DNA and by studying the genetic material it is possible to build a classification of the living world that is based not so much on taxonomic convenience but on phylogenetic facts (Li and Graur 1991; Wallace and Willis 1994). In all form of life, nuclear genomes are large and extremely complex, and nuclear DNA provides almost endless arrays of characters with different structural and functional properties and evolutionary rates (Chen 1995), which in turn can give information on different levels in phylogeny.
A part of the nuclear DNA which is highly repeated, consists of the multigene family coding for the ribosomal RNAs, which is most widely used in phylogenetic analyses (Li and Graur 1991, Avise 1994). In eukaryotes, three of the four RNA components of ribosomes are encoded by a single transcription unit, which is generally tandemly repeated many times. Each transcription unit consists of one copy of each of the three coding regions, 18S, 5.8S and 28S, separated by internal transcribed spacers (ITS1 and ITS2), and an external transcribed spacer (ETS) located upstream of the 18S gene. These transcribed spacers contain signals for processing the rDNA transcript. Adjacent, ribosomal RNA transcription units are separated by a non-transcribed spacer (NTS) or intergenic spacer (IGS) (Figure 2). This region contains subrepeating elements, which enhance transcription (Chen 1995).

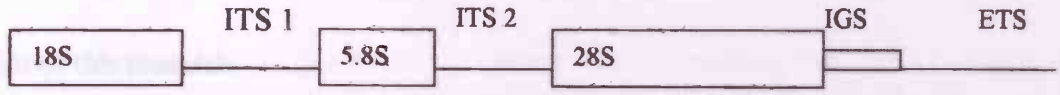

Figure 2. Schematic overview of the nuclear rDNA repeat unit, containing ITS1 and ITS2 regions, which can be found between 18S, 5,8S and 28S rRNA genes. This unit can be found in many repeats in the genome.

Looking at evolution of the multigene family, one would expect that all members of a multigene family would evolve separately. This however, is not the case. In the frog *Xenopus laevis* homogenisation of the gene family encoding for rDNA has been shown within this species (Brown et al. 1972; Futuyma 1998). Thus a multigene families evolves in concert which means that individual members do not evolve independently of the other members of the family. This results in more variation between species – or interbreeding populations – than within one species. Multigene families have a high number of tandem repeats and when studying the evolutionary history it is unlikely that the same mutation could have occurred independently at each locus and being fixed by

selection. A system, which provides homogenisation within this family, must occur. Two mechanisms have been discovered, known as unequal crossing over and gene conversion. Unequal crossing over is a reciprocal recombination process that creates a sequence duplication in one chromatid or chromosome and a corresponding deletion in the other. Gene conversion is a non-reciprocal recombination process in which two sequences interact in such a way that one is converted by the other. Only one chromatid of chromosome sequence is altered (Ridley 1996; Li and Graur 1991; Futuyma 1998). For phylogenetic purposes, nuclear ribosomal sequences can be of great use. Both coding and non-coding regions have been used to investigate phylogenetic relationships from the phylum to the genus level (van Oppen 1993; Odorico and Miller 1997; Avise 1994; Chen and Miller 1996).

Nuclear introns are useful as well and generally accumulate mutations at a much higher pace than the coding regions and they may therefore be useful for comparisons more at the inter and intraspecific level. In contrast with ITS1 and ITS2, the nuclear DNA Pax-C gene is single copy and occurs at only a single locus (Catmull et al. 1998; Galliot 1999). An intron is present at the 5'end of the homeobox of this gene at a position corresponding to residues 46/47 in the homeodomain (Catmull et al. 1998). Apart from a pilot study on a range of Indo-Pacific *Acropora* species (van Oppen, unpubl.), nothing is known to date about the use of Pax-C intron sequences as molecular markers. However, in addition to the rDNA ITS regions the Pax-C intron could provide more information on polymorphism and interspecific variation of the Caribbean *Acropora* species.. Evolution of single copy DNA occurs due to recombination and mutation events. Several types of mutation can be found, among others at a single base like point mutation but also mutations involving whole pieces of chromosomes (Ridley 1996; Futuyma 1998). Through selection and drift, mutations can become fixed in a population. When comparing populations with each other, differences can be found through evolution of these populations, for instance due the lack of gene flow created by specific barriers. Nuclear introns have, compared to coding regions, a higher rate of obtaining mutations, what makes these regions more suitable for studying interspecific relationships.

Aim of this research

In this research the three Caribbean morphospecies, *A. palmata*, *A. cervicornis* and *A. prolifera*, are analysed at the molecular level. These species have evolved independently from the Indo-Pacific *Acropora* species for at least 3 million years (i.e. the closure of the Isthmus of Panama (Kennett 1982). They represent a good model to study evolutionary processes and hybridisation and being a relative small and therefore simple system the results of this study will be useful for comparison with similar data on Indo-Pacific *Acropora* species.. The three species show differences in morphology but the question remains whether these three species represent a single polymorphic species or whether they can be defined as true species. In this research ITS1, ITS2, 5.8S and Pax-C intron sequences were analysed to investigate the questions mentioned above.

Materials and methods

- Sampling corals

Samples of *Acropora palmata*, *Acropora prolifera* and *Acropora cervicornis* were collected on the reefs off Bonaire and Curaçao using scuba by Dr. B.L. Willis (Bonaire) and Onno Diekmann (Curaçao). Samples from Bonaire were from different places along the leeward side of the island: Redslave, Invisibles and Bonaire Beach Bungalows (Table 1). From Curaçao only two species (*A. palmata* and *A. cervicornis*) were collected from Buoy I – 500 m from the research institute Carmabi on the leeward side of the island at 4.0 m, 4.5 m and 5.5 m depth. Samples from Bonaire were taken from colonies of *A. palmata* growing near *A. prolifera* in the high energy zone on the reef crest and samples from colonies of *A. cervicornis* were from a few meters further out and a few meters deeper on the reef slope. In addition these samples were compared with samples, already sampled and sequenced, taken from Panama. These samples were taken from San Blas Island, one sample per species (van Oppen pers comm.).

Table 1. Number of samples taken per reef and per species.

	A. palmata	*A. prolifera*	*A. cervicornis*
Bonaire (3 reefs)	5	5	4
Curaçao (1 reef)	3	-	3
Panama (1 reef)	1	1	1

- DNA-extraction from coral tissue

Part of a branch (1-2 cm) was cut of a stock sample, grounded in liquid nitrogen and added to SE-buffer (3 ml 50°C, see appendix 1) with Proteinase-K (25 ul of 20 mg/ml). The solution was incubated overnight at 50 to 55°C while gently shaking (70 rpm). Then 1750 µl of 4.0 M NaCl and 1 volume (4.750 ml) Chloroform was added and the solution was gently mixed for 15 min., followed by centrifugation (20 min, max 3,500 rpm). The supernatant was transferred to a new tube to which isopropanol (2/3 volume) was added. The solution was kept in freezer (-20°C) for at least 30 min. Centrifugation of the solution was performed for 15 min (15,000rpm) after which it was decanted. The DNA pellet was washed twice with 0.3 ml ice-cold 70% ethanol and air-dried. The pellet was resuspended in 200 ul 1.0x TE (o/n, 4°C).

- Amplification of ITS-regions and Pax-C intron using PCR

For each sample 1 ul of a 1/50 dilution of DNA and 24 ul master mix (see appendix 1) with ACF and ACR-primers for ITS-regions (Gibco BRL, see Table 2 for primer sequences) or AmHD FP1 and RP1 for Pax-C intron (Gibco BRL) were used. PCR-

program BD1BD2 (BD1BD2: 95°C 5 min, 47°C 1 min, 72°C 2 min, 94°C 30 sec, 47°C 30 sec, 72°C 1 min 30 sec, go to step 4 and repeat this 5x, 94°C 30 sec, 52°C 30 sec, 72°C 1 min 30 sec, go to step 8 and repeat this 22x, 72°C 10 min, hold at 4°C or 20°C) was used to amplify the regions. Five ul Loading Dye was added and the samples were run on a 0.8% TAE-agarose gel along with a 1 kb-ladder (4 ul, Promega) to estimate the length of the bands.
Visualisation of the amplified ITS and Pax-C intron regions was accomplished with UV-light using a trans-illuminator. Bands were cut from the gel and purified according to the DNA-matrix gel extraction protocol (Jetsorb, GibcoBrl, Life-technologies). Concentration of DNA was measured using a diode array spectrophotometer at 260 nm.

Table 2. Specific *Acropora* primers and their sequences used in amplification of the ITS and the Pax-C intron regions and colony PCR.

primer	sequence (5'-3')
T7	TAATACGACTCACTATAGGGCGA
SP6	GTATTCTATAGTGTCACCTAAAT
A18F	GAACTTGATCGTTTAGAG
A28R	CTGGTTAGTTTCTCGTCC
AmHdF	TCCAGAGCAGTTAGAGATGCTGG
AmHdR	GGCGATTTGAGAACCAAACCTGTA

- T-vector cloning of ITS-region and Pax-C intron into pGEM®T vector

PCR-products were ligated into the pGEM-T vector following the manufacturer's instructions (Promega). One hundred ul of $CaCl_2$ competent cells (NM500 cells) were added to entire ligation mixture (on ice) and incubated for 30 min. Afterwards a heatshock (42°C, 2 min.) was given and the mixture was added to 900 ul SOC-medium. Incubation of the solution followed, first in a waterbath (37°C, 10 min.) and than in a shaker (37°C, 45 min). Afterwards the solution was spun down (5 min. at 3500 rpm) and the supernatant was decanted (~100 ul remained). The pellet was dissolved and the total volume was pipetted onto a plate (XIA LB-plates, see appendix 1) (o/n incubation at 37°C).

- Automated sequencing of ITS and Pax-C intron-clones by colony PCR

Filtered sterile water (1 ul) was pipetted into PCR tubes and a white colony was lightly touched with a sterile toothpick and rinsed in tube. For ITS, five different colonies were taken per sample. The tubes were placed in the PCR-machine and run under the program 95 (95: 95□C 5 min, hold at 20□C). Afterwards 24 ul mastermix with for the ITS-regions primers T7 and SP6 was added. PCR was performed using the program COL (COL: 95□C 30 sec, 52□C 30 sec, 72□C 40 sec, go to step 1 and repeat 29x, 72□C 2 min, Hold at 22□C). 5 ul LD was added to the sample and loaded on a gel. Gel extraction and purification with the gel extraction kit (Jetsorb, GibcoBrl, Life-

technologies) and concentration measurement of the DNA followed afterwards using a diode array spectrophotometer at 260 nm (value times 50 times dilution).
A total volume of 20 ul, including 4 ul BigDye mix (PE Applied Biosystems), 1 ul 3.3 uM primer (A18F or A28R for ITS and AmHD FP1 and RP1 for Pax-C intron) and a total of 15 ul sample (70-100 ng DNA) plus additional water, sample was ran using the program ABIT in the PCR-reaction (ABIT: 96°C 30 sec, 50°C 15 sec, 60°C 4 min, go to step 1 and repeat this 24x, hold at 4°C). PCR-products were purified according to a Sephadex purification protocol (appendix 2) to remove unincorporated dye-terminators. This purification protocol involved the preparing of the Sephadex (G50 Med) by prewelling in H2O for a minimum of 2 hours. After welling the final concentration of Sephadex beats was adjusted to 50%. One ml of 50% beats (mixed well) was added to a 2 ml column (column in 2 ml eppendorf tubes) and allowed to drip dry (a squeeze with a rubber bulb on top of the column was required to start the flow). The column was spun at 2,500 rpm for 2 min exactly with the hinge outside, after which the sample was loaded in middle of column (column in clean 1.5-ml eppendorf tube) and spun at 2,500 rpm for 2 min exactly. The sample was then dried in a vacuum centrifuge (low setting) for 20 min (do not overdry, check whether sample is dry by flicking the tube). The samples were now ready to be run on a 310 genetic analyser (ABI Prism) automated sequencer, which makes use of fluorescent labels. Elongating strands are terminated when a ddNTP with a fluorescent label is incorporated. All four ddNTP's have different emission wavelengths, which can be separated during gel separation (PE-Applied Biosystems).

- Analyses of sequences

An alignment of the sequences was madet by hand in Sequencher 3.0 (Gene Code Corporation). A phylogenetic analysis (Neighbour-joining bootstrap tree; 100 bootstrap replicates, pairwise-distance) was performed in PAUP 3.1.1. and MEGA 1.02 (Molecular Evolutionary Genetics Analysis) together with bootstrap trees. MEGA was also used to calculate distances of Pax-C intron sequences and a comparison to the Pax-C intron sequences of some Great Barrier Reef *Acropora* species was made. With MacClade 3.05 and Word 98, the files from Sequencher 3.0 were transformed to readable files for Mega 1.02.
With Arlequin 1.1 for population genetic data analysis, AMOVA (analysis of molecular variances) was performed to calculate variances between all samples from Bonaire, Curaçao and Panama of all three species. However, because of differences in numbers of samples from the different regions, variances were in addition calculated for samples from *A. palmata* between Bonaire, Curaçao and Panama, what also was calculated for *A. cervicornis*. In addition between the three species from samples of Bonaire the variances was also determined.

Results

Ninety-nine clones of the three Caribbean *Acropora* morphospecies were sequenced. Length varied between 83 and 100 bp, 110 and 127 bp and 162 and 163 for ITS1, ITS2 and 5.8S respectively. The GC-content (Tables 3a and b) shows an average of 55.4 % for the whole rDNA region. Between the different regions little difference can be found ranging from 45.5 % for ITS1 to 52.5 % for ITS2. The 5.8S rDNA region shows the highest amount of GC with almost 61 %. When looking between different species for the whole region approximately the same amount can be found which is about 55 %. Pax-C intron shows a lower amount of GC contents of 39.3 %.

Table 3a. Mean base frequencies from different regions, all three species and within species for total regions (ITS, ITS2 and 5.8S), ITS1, 5.8S and ITS2 are shown from two *Acropora* groups. The *Acropora* '*aspera*'-group (van Oppen et al., submitted) is from the Great Barrier Reef.

group	***'palmata'***		***'aspera'***	
Region	*G+C*	*Length (bp)*	*G+C*	*Length (bp)*
Total	55.4	355-390		
ITS1	39.1	83-100	30.7- 44.6	66- 85
ITS2	52.2	110-127	44.9-56.9	102-140
5.8S	61.4	162-163	55.5-58.5	152-155
Pax-C intron	39.3	434-461		

	G+C
A.cervicornis	55.1
A. palmata	55.0
A. prolifera	56.0

A number of repeats indicate the occurrence of microsatellites within both ITS regions (see Figs 3A and B). For example, a GA-repeat can be seen which is shared by all three species, from both Bonaire and Curaçao. The other samples from both Bonaire, Curaçao and Panama did not have that GA-repeat. The other microsatellite which can be found in the ITS 1-region is a TCCA-repeat (Figure 3B). This TCCA-repeat shows variation in repeat-number ranging from 1 to 6. This TCCA-repeat shows several sequence repeats shared by all three species from sample sites from both Bonaire, Curaçao and Panama. What can point to more intraspecific variation than interspecific variation.
Furthermore, from the ITS sequences, variation within an individual can be found. In Figure 3A three clones can be observed from *A. cervicornis* (sample no. 28.1, 28.2 and 28.4) showing differences in repeat-number of the TCCA-repeat unit in the microsatellite.

A

```
Ace27.3  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCTTCTATTGACCTATGAGAGAGAG:::::::CCTC
Ace28.1  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG:::::CCTC
Ace28.2  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAAAGGTAGTTCATCATCTTCTATTGACCTATGAGAGAGAG:::::::CCTC
Ace28.4  TCGATCGATGGATCCATCCATCC:::::::::::CACGTGAAAGGTAGTTCATCATCTTCTATTGACCTATGAGAGAGAG:::::::CCTC
Ace28.5  TCGATCGA::::TCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG:::::CATC
Ace35.3  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAAAGGTAGTTCATCATCTTCTATTGACCTATGAGAGAGAG:::::::CCTC
Ace42.4  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAGAG:::CCTC
Apa38.4  TCGATCGA::::TCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATCAGAGAGAGAG:::::CCTC
Apr 4.1  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG:::::CCTC
Apr 4.4  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG:::::CCTC
Ace27.1  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCTATCATCGTATATTGACGTATA:::::::::::TCGTATC
Ace27.4  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATA:::::::::::TCGTATC
```

B

```
Apa38.4  TCGATCGA::::TCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATCAGAGAGAGAG::::CCTC
Ace28.5  TCGATCGA::::TCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG:::CATC
Ace28.1  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG:::CCTC
Ace42.4  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAGA::GCCTC
Apr 4.1  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG::::CCTC
Apr 4.4  TCGATCGATCGATCC:::::::::::::::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACCTATGAGAGAGAGAG::::CCTC
Ace28.4  TCGATCGATGGATCCATCCATCC:::::::::::CACGTGAAAGGTAGTTCATCATCTTCTATTGACCTATGAGAGAGAG::::::CCTC
Apa11.3  TCGATCGATGGATCCACCCATCC:::::::::::CACATGAACGGTAGTCTATCATCGTATATTGACGTATC:::::::::::TCGTATC
Apa13.3  TCGATCGATGGATCCATCCATCC:::::::::::CACGTGAATGGTAGTCTATCATCGTATATTGACGTATA:::::::::::TCGTATC
Ace28.3  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATG:::::::::::TCGTATC
Ace30.1  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATG:::::::::::TCGTATC
Ace44.4  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCTATCATCGTATATTGACGTATA:::::::::::TCGTATC
Apa13.5  TCCATCCATCCATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCACCCTATATTGACCTATA:::::::::::TCCTATC
Apa38.1  TCGATCGATGGATCCGTCCATCCATCC:::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATA:::::::::::TCGTATC
Apa40.1  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATA:::::::::::TCATATC
Apr22.4  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATA:::::::::::TCGTATC
Apr 3.1  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATA:::::::::::TCGTATC
Ace30.2  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATA:::::::::::TCGCATC
Ace44.2  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATA:::::::::::TCGCATC
Apa11.1  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAACGGTAGTCCATCATCGTATATTGACGTGTA:::::::::::TCGTATC
Apa15.5  TCGATCGATGGATCCATCCGTCCATCCATCC:::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATA:::::::::::TCGTATC
Apa38.3  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAACGGTAGTCTATCATCGTATATTGACGTATA:::::::::::TCGTATC
Apa40.3  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATG:::::::::::TCGTATC
Apr21.1  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATG:::::::::::TCGTATG
Apr 4.5  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATA:::::::::::TCGTATC
Ace403T  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCTATCATCGTATATTGACGTATA:::::::::::TCGTATC
Apr414T  TCGATCGATGGATCCATCCATCCATCC:::::::CACGTGAACGGTAGTCCATCATCGTATATTGACGTATG:::::::::::TCGTATC
Apa391T  TCGATCGATGGAATCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATG:::::::::::TCGTATG
Apa394T  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATG:::::::::::TCGTATG
Apa395T  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATG:::::::::::TCGTATG
Apr415T  TCGATCGATGGATCCATCCATCCATCCATCC:::CACGTGAAAGGTAGTTCATCATCGTATATTGACGTATA:::::::::::TCGTATC
```

Figure 3. Sequence data of a number of representative clones, which show differences in the ITS1 (A and B) region. Samples are named based on species (Ace = *A. cervicornis*, Apa = *A. palmata* and Apr = *A. prolifera*, see also appendix 2) names, sample number and clone number. Samples names containing a T are from Panama (Teun van Rheede)

Figures 4 A and B show partial sequences of the ITS2-region. In Figure 4A, a part of the ITS can be seen which shows a TCGGAA region. Variation can be seen in copy number of the TGCC and of one of the A's. The sequence, which shows both TGCC and AA, is shared by Bonaire, Curaçao and Panama samples of all three species. In Figure 4B two different regions can be seen having CCTT followed by a number of A's or no CCTT and having a region of 20 bp (GGTGATCACGCATCTTTGTT). Again this repeat is shared by all three species and all different sites. A difference in number of A's can be seen as well.

A

```
Ace28.2      GCCTGCC::::AATTTTTG
Apa11.1      GCCTGCC::::AATTTTTG
Apa13.5      GCCTGCC::::AATTTTTG
Apa15.1      GCCTGCC::::AATTTTTG
Apa15.5      GCCTGCC::::AATTTTTG
Apa39.2      GCCTGCC::::AATTCTTG
Apr 3.2      GCCTGCC::::AATTTTTG

Ace27.1      GCCTGCCTGCC:ATCTTTG
Ace28.5      GCCTGCCTGCC:ATCTTTG
Ace42.2      GCCTGCCTGCC:ATCTTTG
Ace44.4      GCCTGCCTGCC:ATCTTTG
Ace43.5      GCCTGCCTGCC:ATCTTTG
Apr22.5      GCCTGCCTGCC:ATCTTTG
Apr 3.5      GCCTGCCTGCC:ATCTTTG
Apr414T      GCCTGCCTGCC:ATCTTTG
Ace403T      GCCTGCCTGCC:ATCTTTG

Ace30.1      GCCTGCCTGCCAATTTTTG
Ace42.5      GCCTGCCTGCCAATTTTTG
Ace44.2      GCCTGCCTGCCAATTTTTG
Apa15.2      GCCTGCCTGCCAATTTTTG
Apa38.1      GCCTGCCTGCCAATTTTTG
Apa40.1      GCCTGCCTGCCAATTTTTG
Apa 7.1      GCCTGCCTGCCAATTTTTG
Apa11.3      GCCTGCCTGCCAATTTTTG
Apa13.3      GCCTGCCTGCCAATTTTTG
Apa 7.3      GCCTGCCTGCCAATTTTTG
Apr22.3      GCCTGCCTGCCAATTTTTG
Apr21.3      GCCTGCCTGCCAATTTTTG
Apr 4.1      GCCTGCCTGCCAATTTTTG
Apr 5.3      GCCTGCCTGCCAATTTTTG
Apa391T      GCCTGCCTGCCAATTTTTG
Apa394T      GCCTGCCTGCCAATTTTTG
Apa395T      GCCTGCCTGCCAATTTTTG
Apr415T      GCCTGCCTGCCAATTTTTG
```

Figure 4. Sequence data of several clones which show much difference in ITS2 (A and B) region. Samples are named based on species (Ace = *A. cervicornis*, Apa = *A. palmata* and Apr = *A. prolifera*, see also appendix 2) names, sample number and clone number. Samples names containing a T, are from Panama (Teun van Rheede)

This figure (4B) is continued from the page before

B

```
Ace27.3     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ace28.3     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ace30.2     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ace35.1     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ace42.2     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ace43.5     CCGCCTTAAAAAA:::::::::::::::::TTG::AGTCAGTCA
Ace44.5     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ace42.3     CCGCCTTAAAAAAA::::::::::::::::TTG::AATCAGTCA
Apa11.3     CCGCCTTAAAAAAA::::::::::::::::TTG::AATCAGTCA
Apa13.3     CCGCCTTAAAAAAA::::::::::::::::TTG::AATCAGTCA
Apa40.4     CCGCCTTAAAAAAA::::::::::::::::TTG::AATCAGTCA
Apa 7.3     CCGCCTTAAAAAAA::::::::::::::::TTG::AATCAGTCA
Apr 3.1     CCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Apr22.3     CCGCCTTAAAAAAA::::::::::::::::TTG::AATCAGTCA
Ace27.1     CCG::::AAAAAAAA:GGTGATCACGCATCTTTGTTACTTAGTCA
Ace27.4     CCG::::AAAAAAAA:GGTGATCACGCATCTTTGTTACTTAGTCA
Ace28.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Ace30.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTGCTTAGTCA
Ace35.3     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Ace42.5     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Ace43.1     CCG::::AAAAAAAA:GGTGATCACGCATCTTTG?TACTTAGTCA
Ace44.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa11.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa12.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa13.2     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa15.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa38.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa40.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa39.2     CCG::::AAAAAAA::GGTGATCACACATCTTTGTTACTTAGTCA
Apr 3.2     CCG::::AAAAA::::GGTGATCACACATCTTTGTTACTTAGTCA
Apr415T     CCG::::AAAAAAA::GGTGATCACACATCTTTGTTACTTAGTCA
Apa 7.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apr21.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apr22.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apr 3.5     CCG::::AAAAAAAA:GGTGATCACGCATCTTTGTTACTTAGTCA
Apr 4.1     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apr 5.3     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Ace403T     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa391T     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apr414T     CCG::::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Apa38.4     CCG::::AAAAAAAAAGGTGATCACACATCTTTGTTACTTAGTCA
```

Pax-C intron sequences show 28 sites where variation can be seen. This variation was spread rather evenly over the whole sequence. Three mutations occur in two or more sequences and hence are (potentially) phylogenetically informative. Sequences differing at these sites were assumed to represent different alleles (underlined in Figure 5). Variation in other sites (mutation in only a single sequence) could be due to PCR errors. It is however unlikely that all of those are due to PCR errors because most of the Pax-C intron sequencing was performed by direct sequencing.

```
Apa 7.2     CGCCAGATATATAATGTTTCTATATACC
Apa11       CGTCAGATATATAATGTTTTTATATACC
Apa12       CGCCAGATATATAATGTTTTTATATACC
Apa13.2     CGCCAGATATATAATGTTTTTATATACC
Apa13.1     CGCCAGATATATAATGTTTTTATATACC
Apa15.2     CGCCGCGTATATAATGCTTTTATATACC
Apa15.1     CGCGAGATATATAATGTTTTCATATACC
Apa 7.1     CGCCAGATATATAACGTCTTTACGTACT
Apa382      CGCCAGATATATAATGTTTTTATATACC
Apa38       CGTCAGATATATAATGTTTTTATATACC
Apa39.2     CGTCAGATATATAATGTTTTTATATACC
Apa39.1     CACCAGATATATGATGTTTTTATATACC
Apa39.3     CGCCAGATATATAATGTTTTTATATACC
Apa39.4     CGCCAGA????TAATGTTTTTATATACC
Apa39       CGTCAGATATATAATGTTTTTATATACC
Apa392      CGCCAGATATATAATGTTTTTATATACC
Apa40       CGCCAGATATATAATGTTTTTATATACC
Apa40.1     CGCCAGATATATAATCTTTTTATATATC
Apa40.2     CGCCAGATATAT:ATGTTTTTACATACC

Ace28       CGCCAGATATATAATCTTTTTATATATC
Ace30.1     CGCCAGATATATAATCTTTTTCTACATC
Ace35.1     CGCCAGATATATAATCTTTTTATATATC
Ace40.5     CGCCAGATATATAATCTTTTTATATATC
Ace40.4     CGCCAGATATATAATCTTTTTATATATC
Ace40.3     CGCCAGATATATAATGTTTTTATATACC
Ace42       CGCCAGATATATAATGTTTTTATATACC
Ace422      CGCCAGATATATAATGTTTTTATATACC
Ace43       CGCCAGATATATAATCTTTTTATATATC

Apr 3       CGCCAGATATATAATGTTTTTATATACC
Apr20.2     GGCCAGATATATAGTGTTTTTATATACC
Apr20.1     CGCCAGATATATAATCTTCTTATATATC
Apr21.1     CGCCAGATATATAATCTTTTTATATATC
Apr21.2     CGCCAGATATATAATCTTTTTATATATC
Apr22.1     CGCCAGATATACAATGTTTTTATATGCC
```

Figure 5. Variable sites within the Pax-C intron. In grey, three sites where phylogenetically informative variation could be found. Underlined sequences show two alleles of the Pax-C intron, which can be found in all three species. Other highlighted areas(yellow) show sites where point mutations can be found.

A pairwise distance comparison of the ITS-regions and the 5.8S region shows for ITS1, ITS2 and 5.8S respectively up to 13%, 6.9% and 2.2% variation, using a p-distance analysis (Table 4). For Pax-C intron lower distances are found. Within species between different sampling sites, not much variation can be found either.

Table 4. Uncorrected pairwise distances for the ITS and 5.8S regions separately and all three combined (total) and for the Pax-C intron and in comparison with samples of two *Acropora* groups, '*aspera*' and '*hyacinthus*' the Great Barrier Reef (van Oppen et al., submitted; Márquez pers comm). Pax-C intron with GBR is comparison of Caribbean samples with samples of *Acropora* species from the Great Barrier Reef (see appendix 3). Furthermore distances have also been calculated within species.

Group	***'palmata'***	***'aspera'***	***'hyacinthus'***
Region	*Distance (%)*	*Distance (%)*	*Distance (%)*
ITS1	0 – 13.0	0 - 61.6	13.7 – 55.9
5.8S	0 – 3.1	0 - 11.0	0.6 – 2.6
ITS2	0 – 6.9	0 - 42.2	0.9 – 31.1
total	0 – 5.2	0 - 26.0	0.4 – 17.8
Pax-C intron	0 – 2.2		
Pax-C intron with GBR	0 – 12.5		
A. cervicornis(total)	0 – 5.2		
A. palmata (total)	0 – 4.2		
A. prolifera (total)	0 – 4.9		

The 5.8 S rDNA sequence can be folded according to the secondary structure model, showing stems and loops (Odorico and Miller 1997). The variation in the sequences could be found merely, 20 out of 26 mutations, in the loops, which are not directly involved, in the secondary structure of the 5.8S rDNA gene.

The Neighbour joining p-distance tree based on ITS1 and ITS2 and 5.8S rDNA sequences resulted in a tree with no or hardly any phylogenetic structure (Figure 6). The tree does not show a distinction between the three species. Clusters can be found in which a combination of *A. cervicornis* and *A. prolifera*, *A. prolifera* and *A. palmata*, and *A. cervicornis* and *A. palmata* can be seen. Also the low bootstrap values for a high number of clades indicate no or hardly any phylogenetic structure within this tree.
The phylogenetic tree, which is a neighbour joining p-distance tree (100 bootstrap replications), based on Pax-C intron does not show any structure either (Figure 7).
Comparing the Caribbean sequences of Pax-C with sequences of *Acropora* species from the Great Barrier Reef show a distinct clade for the Caribbean species. Although hardly any structure can be found within this clade (Figure 8).

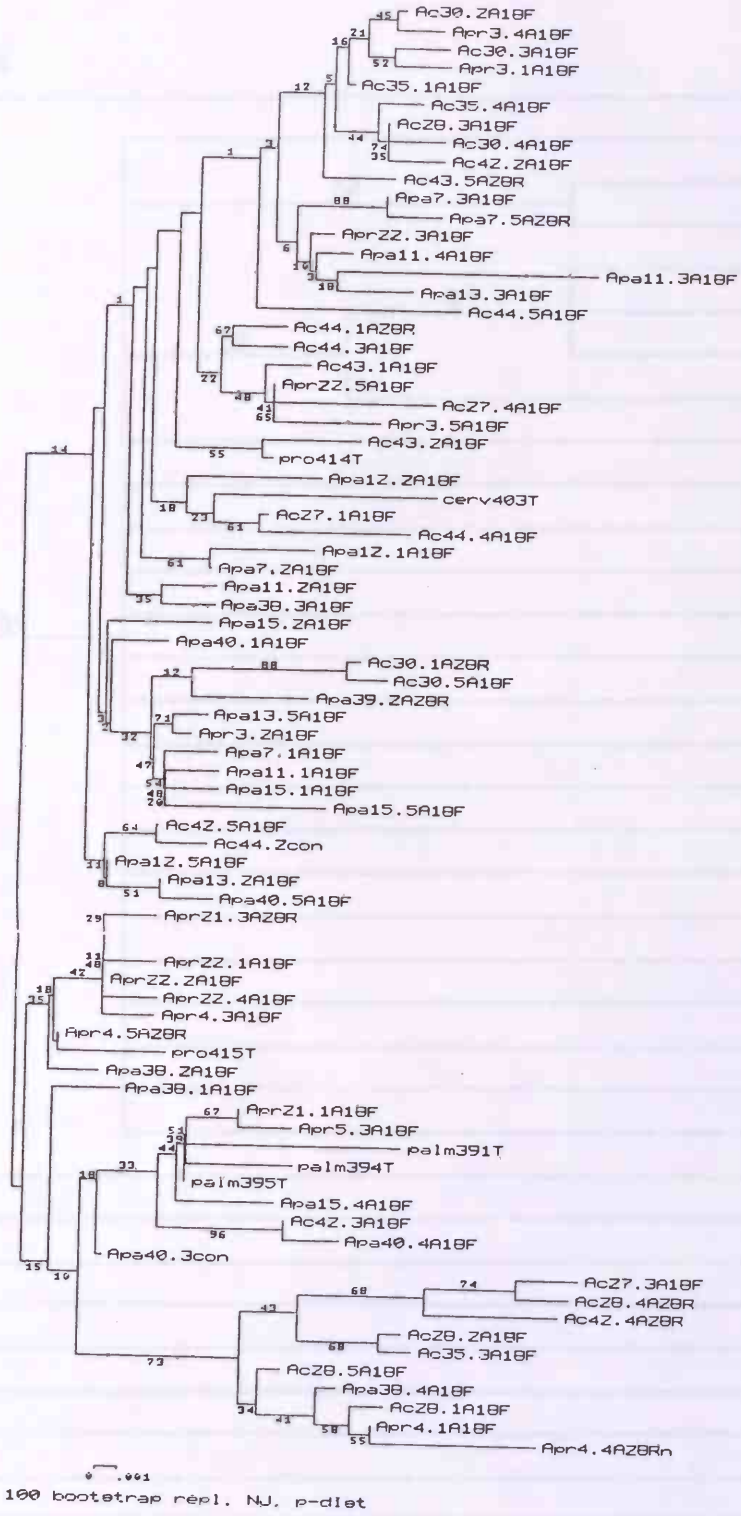

Figure 6. Phylogenetic (neighbour joining, p-distance) tree of Caribbean samples based on ITS1 and ITS2 and 5.8S rDNA sequences (100 bootstrap replications) (For meaning of abbreviations see appendix 2, the samples from Panama have a T in the abbreviation). Numbers below branches indicate bootstrap values.

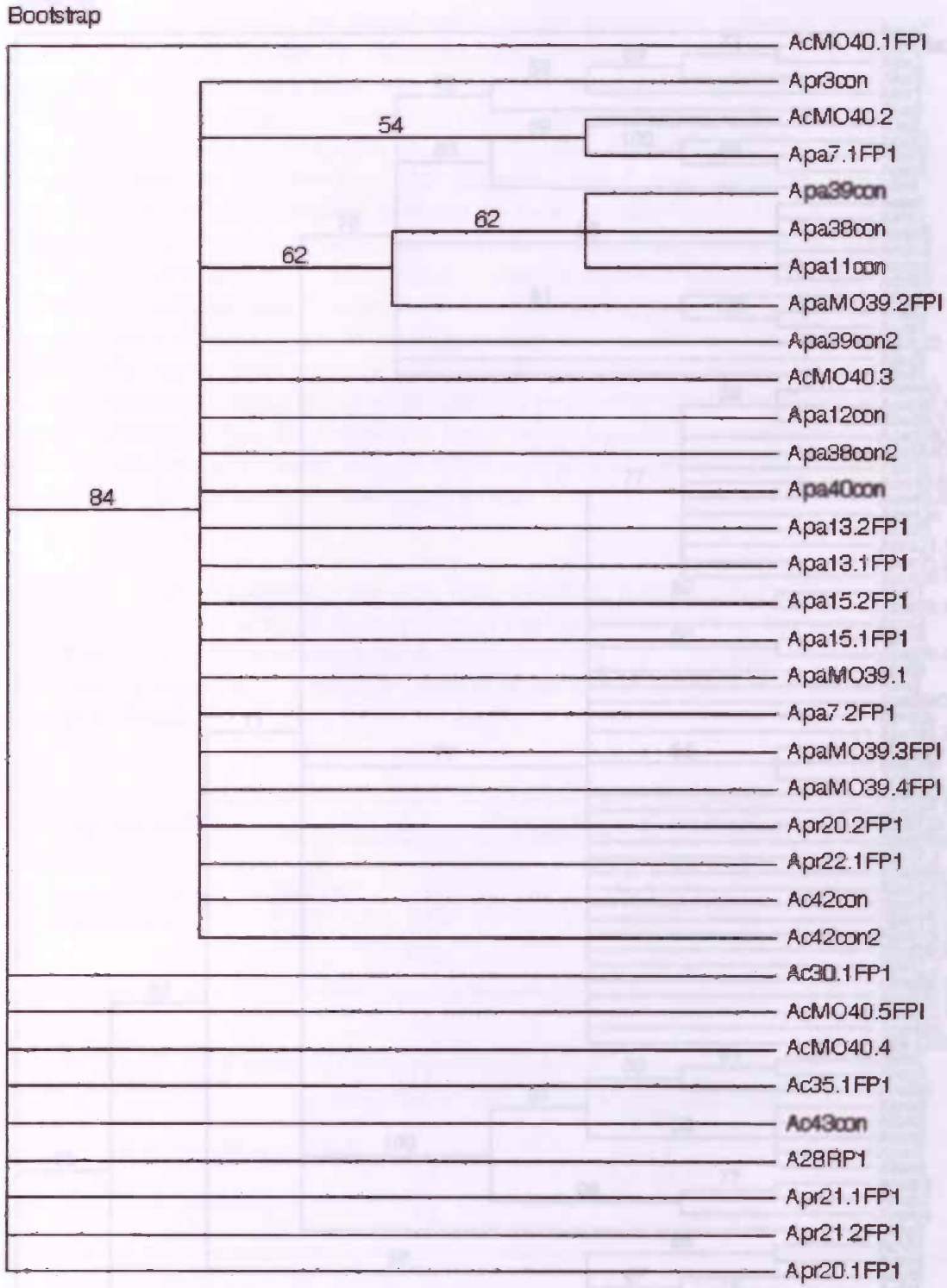

Figure 7 Bootstrap tree. (p-distance n-j, 100 bootstrap replications) of Pax-C intron sequences of Bonaire and Curaçao samples.

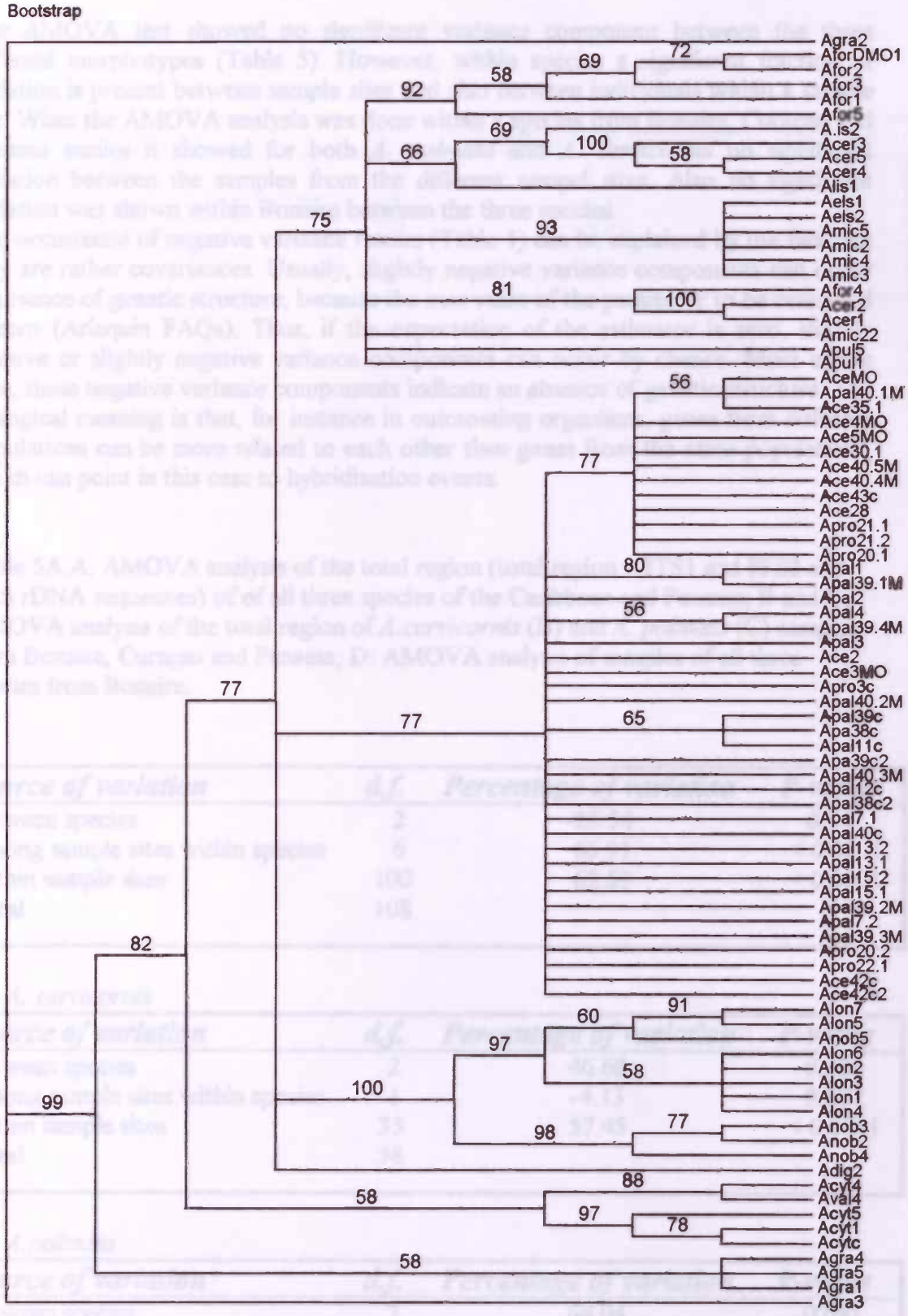

Figure 8 Bootstrap tree (p-distance, n-j tree, 100 bootstrap replications) of Pax-C intron sequences of Bonaire and Curaçao samples in comparison with Great Barrier Reef samples (van Oppen in publ.) For abbriviated *Acropora* names of Great Barrier Reef see Appendix 3).

The AMOVA test showed no significant variance component between the three different morphotypes (Table 5). However, within species a significant fraction of variation is present between sample sites and also between individuals within a sample site. When the AMOVA analysis was done within a species from Bonaire, Curaçao and Panama samles it showed for both *A. palmata* and *A. cervicornis* no significant variation between the samples from the different sampel sites. Also no significant variation was shown within Bonaire between the three species.
The occurrence of negative variance results (Table 4) can be explained by the fact that they are rather covariances. Usually, slightly negative variance components can occur in absence of genetic structure, because the true value of the parameter to be estimated is zero (Arlequin FAQs). Thus, if the expectation of the estimator is zero, slightly positive or slightly negative variance components can occur by chance. Most of the time, these negative variance components indicate an absence of genetic structure. The biological meaning is that, for instance in outcrossing organisms, genes from different populations can be more related to each other than genes from the same populations, which can point in this case to hybridisation events.

Table 5A.A: AMOVA analysis of the total region (total region = ITS1 and ITS2 and 5.8S rDNA sequences) of of all three species of the Caribbean and Panama; B and C: AMOVA analysis of the total region of *A.cervicornis* (B) and *A. palmata* (C) samples from Bonaire, Curaçao and Panama; D: AMOVA analysis of samples of all three species from Bonaire.

A.

Source of variation	***d.f.***	***Percentage of variation***	***P-value***
Between species	2	-28.54	0.747
Among sample sites within species	6	65.91	< 0.0001
Within sample sites	100	62.63	< 0.0001
Total	108		

B. *A. cervicornis*

Source of variation	***d.f.***	***Percentage of variation***	***P-value***
Between species	2	46.68	0.326
Among sample sites within species	1	-4.13	0.887
Within sample sites	35	57.45	< 0.0001
Total	38		

C. *A.palmata*

Source of variation	***d.f.***	***Percentage of variation***	***P-value***
Between species	2	68.04	0.081
Among sample sites within species	2	1.00	0.350
Within sample sites	31	30.96	< 0.0001
Total	35		

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D. Bonaire

Source of variation	***d.f.***	***Percentage of variation***	***P-value***
Between species	2	6.17	0.087
Among sample sites within species	3	-2.60	0.653
Within sample sites	30	96.43	0.283
Total	35		

Discussion

GC-contents

Comparison of the GC-content of species in the *Acropora* '*palmata*' group with those in the *Acropora* '*aspera*' group of the Great Barrier Reef shows that slightly higher amounts are present in ITS regions of the former, although the 5.8S gene of the '*aspera*' group is higher than that of the *Acropora* '*palmata*' group (van Oppen unpublished). This is not against expectations, for related species should show relative comparable GC-content. *Rhodactis* species show similar GC-contents ranging from 45.4 % to 52.5 % in ITS1 regions (Chen and Miller 1996). In comparison, the plant *Zea mais* has much higher GC-content (up to approximately 70 % for ITS1 and ITS2), mosquitoes show only slightly higher contents of 50 % to 58% (Wesson et al. 1992) and the African malaria vector *Anopheles funestus* contains approximately 50 % GC in ITS2 (Mukabyire et al. 1999).

Distance analysis

Uncorrected pairwise proportional distances of the two rDNA ITS –and the 5.8S regions among the three Caribbean *Acropora* species were substantially lower than those between the species in the *A.* '*aspera*' group (van Oppen et al., submitted). In the *Acropora* '*aspera*' group p-distances for ITS1, 5.8S and ITS2 were approximately 62 %, 11 % and 42 % respectively, whilst distances of only 13 %, 3.1 % and 6.9 % were observed in the *A.* '*palmata*' group. The '*aspera*'-group on the Great Barrier Reef consist of more species than the Caribbean '*palmata*'-group. Relations within this group are probably more complicated than in the '*palmata*'-group. In relation to other anthozoan studies on for instance *Rhodactis* species ITS can show high amount of divergence between species (Chen and Miller 1996). In this study *Rhodactis* species from several different regions worldwide, among others the Great Barrier Reef, the Red Sea and the Caribbean Sea, showed an average of 71.85 % between the sequences. Intraspecific similarity observed between samples from was very high (> 98 % on reefs on Great Barrier Reef to 100 % in Eilat and Caribbean samples). *Zea mais* shows even higher amounts of divergence between different species, up to 50-59% (Buckler and Holtsford 1996). In these last two studies, the species examined were distinct from each other as revealed by phylogenetic analyses.
However, studies on the malaria vector *Anopheles funestus* showed for ITS2 almost identical sequences. Although nothing was mentioned on percentage divergence, both mitochondrial *Cytochrome b* and rDNA ITS2 showed a lack difference between isolated chromosomal taxa within this species-complex (Mukabayire et al. 1999). It was thought that this lack is due to recent speciation events. In addition, a recent study on rDNA ITS in the scleractinian coral *M. annularis* showed only 19 variable sites for ITS of which 6 were phylogenetically informative. The whole region was 665 nucleotides long. Nevertheless, none of these sites was fixed within the proposed species (Medina et al. 1999). Based on these results, the *M. annularis* group is now considered as species again.

Pax-C intron and the fossil record of *Acropora* in the Caribbean

The Pax-C intron does not show a high level of variation between these three morphospecies and the phylogenetic analysis reveals hardly any phylogenetic structure. This was unexpected for the Pax-C intron if hybridisation events were assumed not to take place. Nuclear introns have higher rates of mutation accumulation than the coding regions and should show more divergence between than within species due to lineage sorting. Indeed, interspecific difference are large among *Acropora* species from the Great Barrier Reef (van Oppen, unpubl.). Table 4a shows pairwise sequence distances between the Caribbean and some of the Indo-Pacific *Acropora* species. The maximum value of 12.5 % is an underestimation of the real maximum genetic distance among these groups of species, since sequences that created too many gaps within the alignment or were almost unalignable in some regions were left out. The fossil record indicates that *A. cervicornis* dates from the late Pliocene (~ 5 My ago), *A. palmata* from the Pleistocene (~ 1 My years ago) whilst *A. prolifera* is of Holocene origin (last 10,000 years) (Budd et al. 1994, Veron 1995). Whether this history is relatively short compared to the history of the Great Barrier Reef species, is not known. The fossil record of Indo-Pacific species is not very extensive. If the Caribbean *Acropora* species are younger than the Indo-Pacific species, it could explain the lower divergence in the *Acropora* '*palmata*' group. Alternatively, interspecifc hybridisation may explain the differences in divergence of the Caribbean versus Indo-Pacific species groups. The Indo-Pacific *Acropora* species can potentially hybridise with many species (up to 70 species can occur sympatrically and most of those spawn simultaneously), whereas only three *Acropora* species occur in the Caribbean. Occasional hybridisation with very divergent species may result in introgression of extremely different ITS sequences, hence leading to the observed high levels of intraspecific and intra-individual variability at these loci in the Indo-Pacifc *Acropora* species. Interspecific hybridisation may also explain the lack of phylogenetic structure within the Caribbean *Acropora* species. However, it is impossible from the data presented in this study to say whether hybridisation happened in the past or if it is still occurring. Moreover, there is no evidence for *A. prolifera* being an intermediate between *A. palmata* and *A. cervicornis* as expected based on earlier studies (Vaughan 1919). It is possible that *A. prolifera* has originated from hybridisation between *A. palmata* and *A. cervicornis*, but the sequence data suggest that it is able to back-cross with both *A. palmata* and *A. cervicornis* since all three species share similar sequence types. From research on ITS and 5.8 S rDNA sequences of *Acropora* species from the Great Barrier Reef (Odorico and Miller 1997, van Oppen et al. submitted), it has been suggested that the at least some species in the genus *Acropora* exhibit extensive interspecific hybridisation.
Alternatively, the shared presence of several sequence repeats among the three morphospecies may represent an ancestral polymorphism. This is a less plausible explanation because the fossil record shows that the three species are of different age and two of them are relatively old (i.e. *A. cervicornis* is approximately 3 My old and *A. palmata* approximately 1 My old).

Furthermore, other *Acropora* species that used to occur in the Caribbean are *A. panamensis* and *A. saludensis* which both date from the late Oligocene (~ 25 Mya) to the early Pliocene (~ 4 to 5 Mya). With the turnover in Caribbean reef corals during the Plio-Pleistocene about 4-1 Mya, possibly related to changes in climate and oceanic circulation patterns resulting from the closure of the Isthmus of Panama at approximately 3.5 Mya, these species became extinct (Johnson et al. 1995). An explanation given for the modern *Acropora* species according to Frost (1977) could be

that hybridisation between *A. cervicornis* and the extinct *A. pananmensis* have resulted in *A. palmata* (Veron 1995).

Molecular variance of ITS and other research results

The analysis of molecular variance of the ITS-sequence data shows no significant partitioning of genetic variation between species, which is concordant with the results from the phylogenetic analyses. Further analysis of the variation within a species and between the tree species on Bonaire showed no significant variation either. Samples from Bonaire, Curaçao and Panama cluster in the same clades and in addition, clades can be found in which the different sample sites on Bonaire are grouped together.
In contrast to the DNA sequence results from the present study, morphometric studies based on colony and corallite morphology, and reproductive characters, based on size and amount of eggs produced, show that *A. palmata* and *A. cervicornis* are distinct and it was be concluded that they may represent separate morphological species. In this latter research, *A. prolifera* showed to be intermediate between *A. palmata* and *A. cervicornis* and was suggested to be a hybrid between the other two Caribbean *Acropora* species (Stockwell and Willis pers comm).

Evolutionary history

Despite its geological record back to the early Tertiary, *Acropora* does not seem to acquire its modern ecological prominence until several million years ago during the Pliocene and Pleistocene period (Budd 1994). It is possible that new niches became available during the climate changing, allowing speciation via hybridisation to proceed. Evidence for such an evolutionary pathway has been found in angiosperms and ferns (Stebbins 1950; Dawson 1962). High incidence of polyploid species in glacial regions of North Europe has been interpreted as an effect of species from different refugia moving quickly into the land that was newly made available for colonisation, after the retreat of the Pleistocene ice-sheets (Kennett 1982). This form of speciation may also be an explanation for the Caribbean *Acropora* '*palmata*' group. Rapid sympatric speciation involving polyploidy, may have been facilitated in *Acropora* by the availability of new shallow water habitats during sea level changes and changing patterns of ocean circulation (Veron 1995). It is possible that *A. palmata* and *A. cervicornis* have descended from now extinct *Acropora* species then hybridised, resulting in *A. prolifera*. Due to changing environments, new niches became available and *A. prolifera* could have taking position in the back-reef, where it is most abundant today. To test this hypothesis it would be very informative to investigate polyploidity of the *Acropora* species in the Caribbean.

The comparison of *Platygyra* with *Acropora*

Comparable to *Acropora* is the *Platygyra* case. In a study on the coral *Platygyra* on the Great Barrier Reef (Miller and Babcock 1997, Miller and Benzie 1997) a lack of genetic structure was found. No fixed allelic differences were observed at any of the nine investigated allozyme loci between three *Platygyra* species. In addition, no clear-cut habitat in *Platygyra* has been shown, since all morphospecies can be found in various habitats on a single reef and *Platygyra* species are known to participate in the

annual mass-spawning event. Fertilisation trials showed that the production of viable larvae is possible among all seven morphospecies on the Great Barrier Reef. Possible explanations for these observations were hypothesised to be recent speciation due to which no genetic fixation (Miller and Benzie 1997) nor complete reproductive isolation between the morphotypes of *Platygyra* has yet occurred (Miller and Babcock 1997).

In conclusion, ITS and Pax-C intron sequences show a lack of phylogenetic structure between the three Caribbean *Acropora* morpospecies. Several sequence repeats could be found in all three species what points to more variation within than between species and this suggests that reticulation is an important aspect of the evolutionary history of the species in the *A. 'palmata'* group. From the current data set it remains difficult to assess the extent to which hybridisation takes place and whether it occurred in the past or is still occurring at present. It is also not possible to verify that *A. prolifera* originated by hybridisation of *A. palmata* and *A. cervicornis*. Therefore, further research is needed, such as additional *in vitro* hybridisation experiments and investigation of polyploidy of the three species in the *Acropora* '*palmata*'-group. An attempt is being made to study microsatellites in *Acropora* species of the Great Barrier Reef (Márqez, pers. comm.). Microsatelites evolve even quicker than ITS-regions, which could reveal more details on population structure within the *A.* '*palmata*' group. Finally, it would be interesting to find out which genes are involved in morphology and study variation at these loci.

Acknowledgement

I would like to dedicate this thesis to my parents for making it supporting me on several levels during my stay here in Australia, thank you very much!
In addition, I would like to thank the following people for making it possible to perform this research. First of all my supervisors Madeleine van Oppen and David Miller for guiding me and teaching me more about molecular techniques and using the required facilities in the lab and also for having, I had a very good time here in Australia.
The funding of my stay was among others supported by the Marco Polo Fonds, Groninger Universiteits Fonds, Christine Buisman Fonds and the Schimmel Schuurman-van Outeren Stichting and I would like to thank them as well.
Furthermore, I would like to thank dr. Bette Willis, Onno Diekmann and Wiebe Kooistra for the collecting of the samples used in this study. Teun van Rheede I would like to thank for using the sequences from Panama.
Luis Márquez and Madeleine for explaining and helping in performing the methods in this research and allowing me to use additional results on the *Acropora* '*aspera*' and the '*hyacinthus*' group of the Great Barrier Reef. I am also very grateful to Bette Willis and Brian Stockwell for using additional results on morphometrics and reproductive strategies and hybridisation experiments with the Caribbean *Acropora* species.
Finally, I would like to thank Wytze Stam for being my supervisor in the Netherlands and my mother and father and sisters for supported me during good and bad times.

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Appendix

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1. Solutions

- SE-buffer:

3 ml 5 M NaCl
10 ml 0.5 M EDTA (pH 8.0)
5 ml 10 % SDS
182 ml sterile dd H2O

- Tris-HCl pH 8.0 (1 l):

121.1 g Tris-base
800 ml dd H2O
adjust pH using concentrated HCl
top up to 1 l using dd H2O

- 10% SDS pH 7.2 (500 ml):

50 g SDS
400 ul dd H2O
adjust pH with paper only
top up to 500 ul

1. TE-buffer pH 8.0 (1 l):

10 ml 1 M Tris HCl pH 8.0
2 ml 0.5 M EDTA pH 8.0
adjust pH with concentrated HCL
top up to 1 l

- 0.5 M EDTA pH 8.0 (200 ml):

37.22 g EDTA
190 ml dd H2O
adjust pH with NaOH (approximately 5 g needed)
autoclave

- 4 M NaCl (250 ml):

58.443 g NaCl
250 ml dd H2O

- Ethanol (250ml):

100 % ice-cold (-20C):
direct from stock

70% ice-cold (-20C):
70 ml ethanol
30 ml sterile dd H2O

- Isopropanol (250 ml):

Take 250 ml direct from stock and put into –20C
- Chloroform (250ml):

Take 250 ml direct from stock

- Mastermix:

1 ul FP (10 uM)
1 ul RP (10 uM)
2.5 ul dNTP (2 mM)
2.5 ul 10x buffer
2.0 ul MgCl2
0.26 ul Taq-polymerase
14.87 ul sterile filtered H2O
Done in hood

- SOC-medium (100 ml):

2.0 g Bacto-tryptone
0.5 g Bacto-yeast extract
1 ml 1 M NaCl
0.25 ml 1 M KCl
1 ml 2 M Mg2+ stock (1 M $MgCl_2 \bullet 6H_20$ / 1 M $MgSO_4 \bullet 7H_2O$), filter-sterilised
1 ml 2 M glucose, filter-sterilised
97 ml dd H2O

autoclave and cool to room temperature. Add 2M Mg2+ stock and 2M glucose, each to a final concentration of 20mM. Bring to 100 ml with sterile, distilled water. Filter the complete medium through a 0.2 mm filter unit. The final pH should be 7.0

- 2M Mg2+ stock:

20.33 g MgCl2.6H2O
24.65 g MgSO4.7H2O
Add distilled water to 100 ml. Filter sterilise.

- LB-medium (500 ml):

5 g Bacto-Tryptone
2.5 g Bacto-Yeast Extract
2.5 g NaCl
Adjust pH too 7.0 with NaOH

- LB-plates with ampicillin:

Make 1 l LB-medium (for approximately 90 plates):

10 g Bacto-Tryptone
5 g Bacto-Yeast Extract
10 g NaCl
in 950 ml dd H2O, dissolve and adjust pH to 7.0 with NaOH and adjust volume to 1 l. Add 15 g agar just before autoclaving. Autoclave and when medium is removed from autoclave, swirl vigorously to distribute agar evenly over solution. Allow medium to cool to 50C and add:
2 ml ampicillin (50 mg/ml)
2 ml IPTG (0.1 M= 23.6 mg/ul)
4 ml X-gal (20 mg/ml)
Mix by swirling to avoid bubbles. To remove bubbles in the plate, flame the surface of the medium with a Bunsen burner before the agar hardens. Let then harden on bench and put open in hood for 20 min to dry a bit.

2. Sample codes

Code used in this research	Species	Comment (no. or description) B. Willis	No.	Location	Date(sampling)	ITS	Pax-c intron
Apa 1	*A. palmata*	711	1	Redslave	8/8/98		
Ace 2	*A. cervicornis*	641	1	S. Windsock	8/9/98		
Apr 3	*A. prolifera*	637	2	Redslave	8/8/98	x	x
Apr 4	*A. prolifera*	638	3	Redslave	8/8/98	x	
Apr 5	*A. prolifera*	639	4	Redslave	8/8/98	x	x
Apa 6	*A. palmata*	712	2	Redslave	8/8/98		
Apa 7	*A. palmata*	713	3	Redslave	8/8/98	x	x
Ace 8	*A. cervicornis*	722	2	S. Windsock	9/8/98		
Ace 9	*A. cervicornis*	721	3	S. Windsock	9/8/98		
Apa10	*A. palmata*	714	4	Redslave	8/8/98		
Apa11	*A. palmata*	715	5	Redslave	8/8/98	x	x
Apa12	*A. palmata*	626	6	Redslave	8/8/98	x	x
Apa13	*A. palmata*	627	7	Redslave	8/8/98	x	x
Apa14	*A. palmata*	628	8	Redslave	8/8/98		
Apa15	*A. palmata*	629	9	Redslave	8/8/98	x	x
Apa16	*A. palmata*	630	10	Redslave	8/8/98		
Apr17	*A. prolifera*	640	5	Redslave	8/8/98		
Apr18	*A. prolifera*	715	6	Redslave	8/8/98		
Apr19	*A. prolifera*	717	7	Redslave	8/8/98		
Apr20	*A. prolifera*	718	8	Redslave	8/8/98		x
Apr21	*A. prolifera*	719	9	Redslave	8/8/98	x	x
Apr22	*A. prolifera*	720	10	Redslave	8/8/98	x	x
Ace23	*A. cervicornis*	609	4	BBB	9/8/98		
Ace24	*A. cervicornis*	655	5	BBB	9/8/98		
Ace25	*A. cervicornis*		6	BBB	9/8/98		
Ace26	*A. cervicornis*	701	7	Invisible	9/8/98		
Ace27	*A. cervicornis*	702	8	Invisible	9/8/98	x	
Ace28	*A. cervicornis*	703	9	Invisible	9/8/98	x	x
Ace29	*A. cervicornis*	704	10	Invisible	9/8/98		
Ace30	*A. cervicornis*	705	11	Invisible	9/8/98	x	x
Ace31	*A. palmata*	colony H. red		N. Windsock	9/8/98		
Apa32	*A. palmata*	colony J. yellow		N. Windsock	9/8/98		
Apa33	*A. palmata*	colony D. red		BBB	10/8/98		
Ace34	*A. cervicornis*			BBB	10/8/98		
Ace35	*A. cervicornis*			BBB	10/8/98	x	x
Ace36	*A. cervicornis*			BBB	10/8/98		
Ace37	*A. cervicornis*			BBB	10/8/98		
Apa38	*A. palmata*	BI 1		Curaçao		x	x
Apa39	*A. palmata*	BI 2		Curaçao		x	x
Apa40	*A. palmata*	BI 3		Curaçao		x	x
Apa41	*A. palmata*	BI 4		Curaçao			
Ace42	*A. cervicornis*	4.0 m		Curaçao	25/5/98	x	x
Ace43	*A. cervicornis*	4.5 m		Curaçao	25/5/98	x	x
Ace44	*A. cervicornis*	5.5 m		Curaçao	25/5/98	x	x

3. Great Barrier Reef samples

The code of the samples in the tree consist of an abbreviation and a number. The abbreviation is for the *Acropora* species used and the number is the sample used together with the clone. MO, in the Pax-C intron samples of the Caribbean *Acropora* species, stands for Madeleine van Oppen, who sequenced these samples.

Abbreviation	**name**
Apal	*A. palmata*
Apro	*A. prolifera*
Ace	*A. cervicornis*
Apul	*A. pulchra*
Aval	*A. valida*
Alon	*A. longicyathus*
Acer	*A. ceralis*
Alis	*A. listeri*
Aels	*A. elseyi*
Afor	*A. formosa*
Adig	*A. digitifera*
Amic	*A. microphtalma*
Anob	*A. nobilis*
Acyt	*A. cytherea*
Agra	*A. grandis*

4. Sequencing results

ITS 1

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5.8 S region

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Ac35.4A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac35.5A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac42.1A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCAAGCGTCCATCCCCCACGAGGC
Ac42.2A18F      CTCCGCACGGTGGATCTCTCGGCGCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac42.3A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac42.4A28R      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac42.5A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac43.1A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac43.2A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGGGCGTCCATCCCCCACGAGGC
Ac43.3A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac43.4A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Ac44.2con       CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Ac44.3A18F      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa11.1A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa11.5A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa12.1A18F     CTCCGCACGGTGGATCTCTTGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAGCGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa12.2A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa12.5A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa13.2A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa13.3A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa15.3A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa15.5A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGACCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa38.1A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa39.2A28R    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa39.3A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa40.1A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apa40.3con     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa40.4A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa40.5A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa7.1A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa7.2A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa7.3A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa7.4A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apa7.5A28R     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr21.1A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr21.2A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAGCTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr21.3A28R    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr21.4A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr21.5A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr22.1A18F    CTCCGCACGGCGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr22.2A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr22.3A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr22.4A18F    CTCCGCACGGTGGATCTCTCGGCTCACGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr22.5A18F    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr3.1A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr3.2A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr3.3A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr3.4A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr3.5A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACGGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr4.1A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apr4.5A28R     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr5.1con      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGCGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr5.2A28R     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
Apr5.3A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Apr5.5A18F     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
cerv4011T      CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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cerv403T    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGGAGAACGCAACCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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pro4112T    CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
pro414T     CTCCGCACGGTGGATCTCTCGGCTCGCGCATCGACGAAGAACGCAGCCAACTGCGACAGACGTAGTGTGAATTGCAGATCCGATCGTCGATTCTTCGAACGCAAATGGCGCTCGTCTCTCGCGAGGCGAGCAAGGCTGTCCGAGCGTCCATCCCCCACGAGGC
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Ac27.3A18F     GCCTGCCTGCC:ATCTTTGTGGCCGCGTCCTCTGAAGAGCAGGACCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
Ac27.4A18F     GCCTGCCTGCC:ATCTTTGTAGCCGCGTCCTCTGGAGAGCAGAACCG:::AAAAAAAA:GGTGATCACGCATCTTTGTTACTTAGTCA
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Ac28.2A18F     GCCTGCC::::AATTTTTGTGGCCGCGTCCTCTGAAGAGCAGAACCG:::AAAAAAAA:GGTGATCACACATCTTTGTTACTTAGTCA
Ac28.3A18F     GCCTGCCTGCC:ATCTTTGTGGCCGCGTCCTCTGAAGAGCAGGACCGCCTTAAAAAA:::::::::::::::::TTG::AATCAGTCA
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Pax-C intron

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ApaMO39.4FPI
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A42con2
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