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How many came home? Evaluating *ex-situ* conservation of green turtles in the Cayman Islands.

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Abstract

Ex-situ management is an important conservation tool that allows the preservation of biological diversity outside natural habitats while supporting survival in the wild. Captive breeding followed by reintroduction is a possible approach for endangered species conservation and preservation of genetic variability. The Cayman Turtle Centre Ltd was established in 1968 to market green turtle (*Chelonia mydas*) meat and other products and replenish wild populations, thought to be locally extirpated, through captive breeding. We evaluated the effects of this reintroduction program using molecular markers (13 microsatellites, 800bp D-loop and STR mtDNA sequences) from captive breeders (N=257) and wild nesting females (N=57) (sampling period: 2013-2015). We divided the captive breeders into three groups: founders (from the original stock), and then two subdivisions of F1 individuals corresponding to two different management strategies, cohort 1995 ("C1995") and multicohort F1 ("MCF1"). Loss of genetic variability and increased relatedness was observed in the captive stock over time. We found no significant differences in diversity among captive and wild groups, and similar or higher levels of haplotype variability when

compared to other natural populations. Using parentage and sibship assignment, we determined that 90% of the wild individuals were related to the captive stock. Our results suggest a strong impact of the reintroduction program on the present recovery of the wild green turtle population nesting in the Cayman Islands. Moreover, genetic relatedness analyses of captive populations are necessary to improve future management actions to maintain genetic diversity in the long term and avoid inbreeding depression.

Introduction

Over the past two decades, biodiversity loss has become a pressing global issue (Barnosky et al., 2011; Dirzo & Raven, 2003; Hooper et al., 2012; Mora & Sale, 2011). Deforestation (Barlow et al., 2016; Gibson et al., 2013; Turner, 1996), overexploitation (Coleman & Williams, 2002), agricultural expansion (Allan et al., 2015) and invasive species (Ceballos & Ehrlich, 2002; Doherty, Glen, Nimmo, Ritchie & Dickman, 2016) are some of the effects driving species and populations to experience severe decline and negatively influences the functionality of food webs (Dunne, Williams & Martinez, 2002) and ecosystem sustainability (Hooper et al., 2012; Worm et al., 2006).

Ex-situ strategies (i.e. conservation measures applied away from the natural habitat of the target species) such as captive breeding and reintroduction have become an important conservation tool used to combat biodiversity loss by recovering locally extinct populations (Fischer & Lindenmayer, 2000; Storfer, 1999). The release of captive-bred individuals into the wild has been identified as an instrument for conservation of threatened populations (reintroduction) and for the establishment of new ones (introduction) (IUCN 1987). Captive breeding programs followed by reintroductions, although controversial (Jule, Leaver & Lea,

2008), are one of the most commonly used *ex-situ* conservation strategies (Fischer & Lindenmayer, 2000). Captive breeders may include local individuals and/or individuals belonging to other wild populations, depending on the status of the population to be recovered. Some species of conservation concern, such as the Przewalski horse (*Equuferus przewalskii*) in Mongolia or the Yellow-shouldered Amazon Parrot (*Amazona barbadensis*) in Margarita Island (Venezuela) have successfully recovered to self-sustaining populations after captive breeding and reintroduction programs (Sanz & Grajal, 1998; Van Dierendonck, Bandi, Batdorj, Dügerlham & Munkhtsog, 1996). Reintroductions from captive breeding programs may, however, produce individuals incapable of long-term survival in the wild due to feeding incompetence (i.e. incapacity of hunting or finding food resources in the natural habitat), unsuccessful predator/competitor avoidance and disease (Jule et al., 2008). During the 1990s, several studies highlighted the need for monitoring after the release of individuals (Armstrong, Soderquist & Southgate, 1994; Sarrazin & Barbault, 1996; Sutherland et al 2010), and that this monitoring should be driven by key questions to improve efficiency on active conservation (Nichols & Williams, 2006). Nonetheless, outcomes are still often unknown and causes of failures are rarely understood (Rees et al., 2016; Weeks et al., 2011) as a result of the paucity of monitoring and/or the time lag necessary to detect actual failure/success (Fischer & Lindenmayer, 2000).

The origin and number of breeders in *ex-situ* conservation programs should be considered to reduce potential negative impacts during reintroductions, such as the generation of weak hybrid offspring as a consequence of outbreeding depression (Edmands, 2007; Weeks et al., 2011; Witzenberger & Hochkirch, 2011) or the loss of genetic variability and inbreeding depression due to a low number of founders (Hedrick & Fredrickson, 2008; Hedrick, Miller,

Geffen & Wayne, 1997; Ralls & Ballou, 1986; Witzenberger & Hochkirch, 2011). Reintroduction programs may have a differential success, ranging from total failure to complete replacement by reintroduced individuals and extirpation of the wild local population (Sweeting, Beamish, Noakes & Neville, 2003; Sutherland et al., 2010).

Monitoring reintroduction programs can be challenging, in particular for species with high dispersal rates and long generation times (Canessa et al., 2016); therefore several methodologies, from tracking using electronic devices to the use of biological markers, have been adopted in different species. Telemetry was used to monitor dispersal patterns of an endangered freshwater fish (the trout cod *Maccullochella macquariensis*) in Australia (Ebner & Thiem, 2009), whilst growth rates and survival indices were used in the management of the reintroduced peninsular bighorn sheep (*Ovis canadensis*) in California (Ostermann, Deforge & Edge, 2001). Nuclear genetic markers, such as microsatellites, have been valuable for assessing the effectiveness of reintroduction programs and measuring their impact on natural populations (DeMay, Becker, Rachlow & Waits, 2017; Koelewijn et al., 2010; Stenglein, Waits, Ausband, Zager & Mack, 2010). Similarly, mitochondrial DNA (mtDNA) has been successfully used to monitor reintroduction (Godoy, Negro, Hiraldo & Donazar, 2004) and captive breeding programs (Kitanishi et al., 2013). Moreover, combining different types of genetic markers is advantageous to obtain diverse and complementary information about the same sample set (Kim et al., 2011; Puckett et al., 2014).

To date the long term Cayman Turtle Farm (CTF) green turtle (*Chelonia mydas*) reintroduction program has not been evaluated genetically. Green turtles play an important ecological role in the maintenance of seagrass beds, as grazing stimulates new growth (Aragones, Lawler, Foley & Marsh, 2006). The large nesting population of green turtles historically present in the Cayman Islands served as a key fishery resource (Aiken et al., 2001; Bass, Epperly & Braun-McNeill, 2006), and was exposed to massive anthropogenic perturbations by the commercial harvesting of nesting females for meat consumption. The decline of green turtle nesting populations worldwide led this species to be listed as Endangered in 1975 by IUCN (International Union for Conservation of Nature) and its commercialization regulated by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Aiken et al., 2001; Seminoff, 2004). In the 1980s several studies concluded that the green turtle nesting population of the Cayman Islands was extinct (King, 1982; Stoddart, 1980), although, the presence of some green turtles was reported in the waters surrounding the Islands (Brunt & Davies, 2012). In 1968 a private company, the Cayman Turtle Farm (up to 1983 known as Mariculture Ltd. and now called Cayman Turtle Centre Ltd), started a green turtle captive breeding program to restore a population nesting in the Cayman Islands whilst providing an alternative source of turtle meat to alleviate harvest from the wild population (Cayman Turtle Farm, 2002). The project consisted of importing adult turtles and eggs from other populations to breed in captivity, and raised future generations for the reintroduction and as a harvest resource. Individuals representing the F_1 generation were typically grown in the farm up to 4-6 years and then arbitrarily chosen to be part of the breeding stock, to be released or to be slaughtered for meat consumption. On the other hand, individuals of the F_2 generation were only released or used as a source of meat, but not as part of the captive breeding stock.

Adult turtles and eggs were collected from the nesting populations of Costa Rica, Suriname, Guyana, Ascension Island and Mexico and from the foraging area of Nicaragua between 1968 and 1978 to form the founder stock of the CTF (Figure 1; see Supplementary Table S1 and S2). Given that individuals imported to the farm were gathered from widely separated areas, they likely belonged to genetically different populations, as shown by a recent study (Naro-Maciel et al., 2014). Between 1980 and 2001 the CTF released ~30,000 captive-raised hatchlings and yearlings as part of the reintroduction program (Bell et al., 2005). Between 1982 and 1983, the farm reduced the size of the founder breeding stock by 46% (Supplementary Table S3) to decrease management costs (Cayman Turtle Farm, 2002). In 2001, Hurricane Michelle caused major damages to the CTF further decreasing the number of founder breeders with the loss of 81% of individuals, which either died or escaped (Supplementary Table S3).

The reintroduction of marine turtles into the wild is based on the premise that they exhibit natal philopatry. This behavior is described as the return of individuals to their natal site to reproduce (Cury, 1994; Greenwood, 1980; Mayr, 1963). Newborn hatchlings are thought to memorize different chemical and magnetic cues from the nesting beaches where they hatch and use this information in adulthood to find their natal nesting beaches to reproduce (Meylan, Bowen & Avise, 1990; Lohmann, Witherington, Lohmann & Salmon, 1997; Lohmann, Lohmann, Brothers & Putman, 2013). Natal philopatry leads to genetic distinctiveness of populations, thus geographically distant groups might have limited genetic exchange (Chesser, 1991; Lee, Luschi & Hays, 2007). For this reason, the natural recovery of isolated populations on the verge of extinction may be difficult, as little migration would be expected from other populations to increase the number of mating adults. This philopatric

behavior, both in females and males (Clusa et al., 2018), is the basis for a rapid colonization of new potential nesting areas after the first arrival of marine turtles (Carreras et al., 2018).

Philopatry is also the basis of the success of the reintroduction program of Kemp's ridley sea turtle (*Lepidochelys kempii*) in Texas, through a headstarting program started in 1978 (Fontaine, 2005). Headstarting consists of the rearing of the offspring in captivity up to a certain size before their release, to prevent high rates of mortality typical of the early stages of life (Heppell, Crowder & Crouse, 1996; Mitrus, 2005). However, headstarting, as a conservation measure, has been questioned over the last 20 years because of the expected poor survival of the released turtles. They have been found to have nutritional deficiencies and behavioral modifications resulting from factors associated with captivity, including insufficient exercise, lack of stimuli or lack of feeding skills (Heppell, 1998; Heppell et al., 1996; Moll & Moll, 2000). Furthermore, the probability of surviving to adulthood increases exponentially with age, and therefore the population dynamics of organisms such as turtles are driven more strongly by changes in annual juvenile survival than by survival in their first year of life (Heppell et al., 1996).

The headstarting reintroduction program of the Cayman Turtle Farm has also raised some concerns about its utility and possible negative impacts. Some of these concerns are related to human health, animal welfare and conservation activities (Warwick, Arena & Steedman, 2013). Moreover, reintroduction programs have also possible genetic consequences such as the alteration of genetic variability of natural populations caused by the introduction of hatchery bred individuals (Horreo, de la Hoz, Pola, Machado-Schiaffino & Garcia-Vazquez, 2012). Farm releases in the Caribbean region of individuals hatched from a founder stock

that includes South Atlantic genetic material are thus a potential source of outbreeding depression (Narum, Arnsberg, Talbot, & Powell, 2007). Despite these concerns, the wild population of green turtle nesting in the Cayman Islands has increased and the number of nesting females is increasing despite the long generation time of the species (Aiken et al., 2001; Cayman Islands DoE unpublished data). To date, the exact role of the CTF breeding program in this recovery is unknown, but the application of living tags (created by the transplantation of a 4mm diameter disc of plastron to the carapace) has shown that at least some of the released hatchlings survived to adulthood and reproduced on nesting beaches in the Cayman Islands (Bell et al., 2005).

Genetic evaluation and monitoring of the success of the CTF reintroduction program is necessary to understand its contribution to the recovery of the wild populations and its impact on the local gene pool. Using a set of 13 microsatellites, a fragment of the D-loop mtDNA (800 bp) and four mitochondrial simple tandem repeats (STR) markers, we analyzed the genetic diversity and genetic structure of 257 captive and 57 wild green turtle females nesting on the islands of Grand Cayman. This study aims to reconstruct the farm population structure and evaluate the reintroduction programme, specifically 1) estimate the genetic diversity of the farm breeding stock, 2) assess parentage and sibship relationships between farm and wild population and 3) identify the genetic structuring of the farm breeding stock and of the wild population in relation to other wild green turtle populations. We aim to provide novel insights and guidelines for future reintroduction actions using our results as a case study.

Materials and Methods

Sampling and DNA extraction

The study was conducted using samples from wild green turtle females nesting on Grand Cayman (Cayman Islands) and from breeding females of the Cayman Turtle Farm (CTF). Tissue biopsies were taken from all females of the farm breeding stock from 2012 to 2014 (N=257) and from all wild nesting females encountered during 2013 and 2014 (N=57). Tissue samples were taken from the neck or from the rear flippers with a scalpel blade and stored in 100% ethanol. All individuals were PIT tagged (Passive Integrated Transponder) (Bjorndal, Reich & Bolten, 2010) to avoid pseudoreplication. We also obtained information about the origin of the farm breeders or year of birth from the farm databases when available (Supplementary Dataset S1), which indicated that the breeding stock consisted of original founder and captive F_1 individuals. Based on this background data, we identified three sample groups within the farm breeding stock, as they represent different stages of the reintroduction: founders, C1995 and multicohort F_1 breeders (MCF_1). The group 'Founders' includes individuals known to belong to the original stock and to come from distinct populations (N=25). The group 'C1995' consists of F_1 individuals born in the farm in 1995 and kept to increase the number of breeders after hurricane Michelle (N=189). The group 'MCF_1' (Multicohort F_1 breeders) are F_1 females born from 1986 up to 2002 and used for routine replacement of the original founder stock in order to maintain management census sizes (N=43). These two F_1 groups were considered separately because they are the result of two different management strategies (a single large replacement, the first, *versus* continuous small replacements, the latter).

The DNA of all samples was extracted using the QIAamp Blood and Tissue Kit (Qiagen®) or using E.Z.N.A.® Tissue DNA kit (OMEGA Bio-tek), following the manufacturer protocols. DNA was suspended in 100 µL of deionized water.

Laboratory analysis

All samples were genotyped at 13 microsatellite loci, originally designed for different species of sea turtles that amplify and are polymorphic in green turtles (Wright et al., 2012). Additionally, we sequenced an 800bp fragment of the mtDNA D-Loop region (Abreu-Grobois et al., 2006) and four $(AT)_n$ mtDNA STRs (Tikochinski et al., 2012) in all wild individuals and a selection of the farm animals. The selection of farm samples was based on the known origin of the animals coupled with our microsatellite results, in order to characterize the founder stock and to confirm parentage assignments (see results). Amplification PCR conditions for each marker are in Supplementary Table S4.

One of the primers for each microsatellite (Supplementary Table S5) was labeled with a fluorescent dye (6-FAM, HEX or NED). Microsatellite loci were amplified with two multiplex PCR sets as described in the literature (Wright et al., 2012) and carried out with a GenAmp PCR System 2700 (Applied Biosystems®). Each multiplex was amplified in a final volume of 5 µL, with 2.5 µL of Multiplex PCR Master Mix (Qiagen®), 1.5 µL of primer mix (as detailed in the Supplementary Material in Bradshaw et al. 2018) and 1 µL of DNA. After amplification, 15 µL of ultrapure H_2O Ecolab were added in each reaction tube and amplification success assessed in an agarose gel. Microsatellite allele sizes were estimated in 2 µL of diluted amplified DNA, 0.5 µL of GeneScan™ 500 Liz Size standard (Applied Biosystems) and 12.5 µL of deionized formamide on an ABI 3730 DNA Analyzer (Applied Biosystems) at the Serveis

Científico-Tècnics of the Universitat de Barcelona, and alleles assigned using GeneMapper® software (version 3.7, Applied Biosystems). In order to check for genotyping errors 27 samples were randomly selected and genotyped twice, resulting in a genotyping error < 0.2%.

Mitochondrial D-Loop sequences (800 bp long) were amplified in 142 individuals (Supplementary Dataset S1). The final reaction volume was 15 µL containing 5.08 µL of deionized water, 3 µL of PCR buffer 5x (GoTaq® Promega), 1.8 µL of dNTPs (1mM), 0.6 µL of $MgCl_2$ (25mM), 1.8 µL of BSA, 0.3 µL of forward primer (10 µM), 0.3 µL of reverse primer (10 µM), 0.12 µL of GoTaq® G2 Flexi DNA Polymerase (Promega 5u/µL), and 2 µL of DNA. Mitochondrial STRs amplifications were conducted for the same individuals (Supplementary Dataset S1). The final reaction volume was 15 µL, with 5.48 µL of deionized water, 3 µL of PCR buffer 5x (GoTaq® Promega), 1.8 µL of dNTPs (1mM), 0.6 µL of MgCl2 (50mM), 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 0.12 µL of GoTaq® G2 Flexi DNA Polymerase (Promega 5u/µL), and 2 µL of DNA. The amplified DNAs (3 µL) of both mtDNA markers were purified with Exo-SAP (2 µL containing 0.4u of EXO and 0.4u of TSAP) using a single cycle of 37°C for 15 min and 80°C for 15 min. Then, 1 µL (5 µM) of the corresponding forward primer was added to the purified product (LCM15382 for D-loop and CM-D-1 for STRs) and dried at 80°C for 30 min in order to be sequenced on an ABI 3730 automated DNA analyzer (Applied Biosystems) at the Serveis Científico-Tècnics from Universitat de Barcelona.

Data analysis: Microsatellites

We checked for null alleles using the program MICRO-CHECKER (Van Oosterhout, Hutchinson, Wills & Shipley, 2004). Pairwise linkage disequilibrium and deviation from Hardy-Weinberg equilibrium were assessed using GENEPOP v4.3 software (Raymond & Rousset, 2004). In order to correct for multiple comparisons, we used the Benjamini-Yekutieli (B-Y) FDR (False Discovery Rate) correction (Narum, 2006). To test for inbreeding through observed (H_o) and expected (H_e) heterozygosity we used GENETIX v4.05.2 (Belkhir, Borsa, Chikhi, Raufaste & Bonhomme, 2004) software on all groups of individuals. Allelic richness was computed with rarefaction using R package DiveRsity (Keenan, Mcginnity, Cross, Crozier, & Prodöhl, 2013). Pairwise genetic distances (F_{ST}) among our groups were calculated using GENALEX 6.503 (Peakall & Smouse, 2012). We used the same program to calculate the relatedness estimator of Lynch and Ritland (1999) among individuals within each group. Since we are analyzing samples with potentially very distinct origin, we estimated relatedness values for each subset separately based on the allele frequencies obtained within the subset. We also tested if relatedness values for each subset significantly deviate from those randomly obtained by 9999 permutations, as implemented in GENALEX 6.503 (Peakall & Smouse, 2012). For this last analysis we considered all the samples together to calculate baseline allele frequencies.

We identified the most probable number of genetic groups among the founder individuals of the captive stock, since they could come from geographic distant areas, while the rest of samples from the breeding stock belongs to the F_1 of the captive breeding and therefore are the result of a mix of genetic material. We used the software STRUCTURE v2.3.4 (Pritchard, Stephens, & Donnelly, 2000) and performed 10 repetitions of each independent K value

from 1 to 10; burn-in length was set to 50,000 with 500,000 Markov chain Monte Carlo (MCMC). To select the best K we calculated the log probability of the data with STRUCTURE HARVESTER (Earl, 2012).

We conducted sibship and maternity analysis using three different programs based on maximum-likelihood: COLONY v2.3 (Jones & Wang, 2010), CERVUS v3.0.7 (Marshall, Slate, Kruuk & Pemberton, 1998) and ML-Relate (Kalinowski, Wagner & Taper, 2006). COLONY performs parentage assignment, sibship analysis and reconstructs genotypes of unsampled parents. COLONY also generates the best cluster in which the program infers unsampled mothers and fathers and allows forming family groups. We set the parameters to long run, high precision and error rate = 0.0001. All individuals were included as offspring and motherhood input data. To refine the analysis and to minimize the error we excluded as mothers all wild individuals, as they could not have sired any of the farm individuals, and all captive individuals born in 1995 (cohort C1995) or later, according to the information provided by the CTF (Supplementary Dataset S1), as they would be too young to be mothers of the other breeding females. CERVUS performs parentage analysis using strict confidence level set at 95%. ML-Relate estimates the relationship among individuals from codominant genetic data. We computed the log-likelihood of relatedness for all pairs of individuals and produced a confident interval of 95% after 999 simulations per test. The outputs of the three programs were then combined to identify for maternity and sibship relationships in our sample set. Maternity outputs of CERVUS and COLONY were also compared using PedAgree (v1.06), software which can be used to assess accuracy and congruence for genetically reconstructed pedigree relationships from these two programs (Coombs, Letcher & Nislow, 2010).

Data Analysis: Mitochondrial DNA

D-Loop sequences were aligned, cut and compared with published haplotype sequences found in the database maintained by the Archie Carr Center for Sea Turtle Research (http://accstr.ufl.edu/) using BioEdit software (Hall, 1999). STRs were scored by counting the number of $(AT)_n$ repeats in each of the 4 loci of the sequence described in the literature and haplotypes named using the four-number barcoding system (Tikochinski et al., 2012). MEGA7 software (Kumar, Stecher & Tamura, 2016) was used to create a Neighbor-Joining tree (Saitou & Nei, 1987) to identify the phylogeny of D-Loop haplotypes by maximum likelihood (Tamura, Nei & Kumar, 2004) with 999 bootstrap replicates. The tree was rooted in the middle of the longest branch. We also created a haplotype network using Median Joining calculation (Bandelt, Forster & Röhl, 1999) as implemented in NETWORK 5.0 software (www.fluxus-engineering.com). Each D-Loop haplotype was assigned to a lineage by comparison to the lineages identified by Naro-Maciel et al. (2014). We calculated haplotype and nucleotide diversity for wild and captive individuals separately using Arlequin 3.5 (Excoffier & Lischer, 2010) and DnaSP v5 (Librado & Rozas, 2009). Both D-loop and STR sequences were also used to confirm maternal assignments resulting from microsatellite analysis. Using D-loop sequences we performed analysis of wild and captive populations compared to other wild populations of the Caribbean (Shamblin et al., 2015a), African (Patrício et al., 2017; Shamblin et al., 2015b) and Mediterranean (Bradshaw *et al.* 2018) regions. Incorporating D-loop sequences from these populations, we tested for genetic structuring (F_{ST}) and genetic diversity.

We performed Mixed-Stock analysis (MSA) of the Founders subset against a baseline of rookeries with short (400bp) D-loop sequences, consisting of North Caribbean, South Caribbean and South Atlantic populations (23 populations in total). We used short sequences to be able to include data from populations of known origin of the founders according to CTF background records. We used BAYES program (Pella & Masuda, 2001), with 40,000 MCMC runs for each potentially contributing nesting site with prior expectations of 0.978 for a particular nesting site and 0.001 for the twenty-two other nesting sites. Lack of convergence was assessed with the shrink factor of Gelman & Rubin (1992).The contribution of each rookery to the founder group was estimated from the mean of chains after 20,000 burn-in steps.

The results of both mitochondrial DNA and microsatellite analysis of wild and captive were used together to define the farm population structure. For this purpose, we combined D-Loop and STRs in a haplotypic system as in Shamblin et al. 2015b to perform F_{ST} tests among all groups.

Results

Genetic Diversity

All 314 individuals were genotyped with the 13 microsatellite markers showing a number of alleles ranging from 6 (B123) to 23 (Cc2) (Supplementary Table S5). Four markers were found to be in Hardy-Weinberg equilibrium, one (D2) was not at equilibrium in both groups, and the remaining eight yielded different results depending on the sample group considered. However, we decided not to discard any of the markers due to being out of Hardy-Weinberg equilibrium for two reasons. Firstly, previous studies concluded that none of these markers

deviate from Hardy-Weinberg Equilibrium in other wild populations (Bradshaw et al., 2018; Wright et al., 2012), suggesting that the detected deviations are not due to the properties of the marker. Secondly, these deviations are expected to be found both in the captive individuals, considering the process of founding from different natural populations, and in the wild population, due to the reintroduction process. Furthermore, analyses were run without these markers and the results did not change substantially. Expected heterozygosity (H_e) decreased from founders to C1995 and from C1995 to wild individuals (Table 1), although differences were not significant as assessed with a Wilcoxon matched pairs test. Mean observed heterozygosity (H_o) had its highest value on individuals in cohort C1995 although differences were not significant. We obtained a total of 17 D-Loop haplotypes (Figure 2, Supplementary Table S6). All but one had been previously described in populations in the Caribbean Sea, South America, South Atlantic (Ascension Island (Formia et al., 2006)) and Africa (Shamblin et al., 2015a; Shamblin et al., 2015b). The haplotypes found in our samples belonged to different lineages as defined in the literature (Naro-Maciel et al., 2014): most haplotypes belonged to the A lineage (84%), which is typically found in the Caribbean, while the rest belonged to lineage B (Figure 2) typically found in South America, South Atlantic, and Africa (Shamblin et al., 2015a; Shamblin et al., 2015b; Patricio et al., 2017). CM-A5.1 is the only haplotype of lineage B found shared by both the captive and wild populations; however, this haplotype is not exclusive of the South Atlantic region and can be found in other wild populations of the Caribbean region (Naro-Maciel et al., 2014). The new haplotype (CM-A78.1) (Genbank Accession Number: MH177873) belongs to Lineage A (Figure 2). Haplotype diversity (H) and nucleotide diversity (π) decreased from the founder generation to cohort 1995 and to wild females (Table 1). We found 23 different STR haplotypes, with the highest haplotype diversity in the founder generation. When

considering the two mitochondrial markers together, 32 haplotypes were obtained (Supplementary Table S6), also with the highest diversity in the founder generation (Table 1).

Relatedness reconstruction among wild and captive individuals

Individuals of the original founder stock (N=25) showed the lowest degree of relatedness (r=-0.021), while the cohort 1995 (N=189) showed the highest value (r=-0.003, Table 1). Only C1995 presented relatedness values significantly higher than those expected considering the permutation analysis (p = 0.0001).

COLONY identified a total of 82 mothers and 54 fathers (both assigned and inferred), differentially contributing to F_1 generation (Supplementary Figure S1), while almost all founder individuals were sired by different males and females. Mother/father sex ratio proportions for parents assigned to each subset increasing from Founders (sex ratio = 0.96), to MCF_1 (sex ratio = 1.25) to C1995 (sex ratio = 1.52). The proportion offspring/mother (Founders = 1.05, MCF_1 = 1.22 and C1995 = 2.82) and offspring/father increased in the same way, in accordance with the increase of relatedness levels in each group. A total of 43 mothers and 34 fathers were identified for wild females with a sex ratio similar to MCF_1 individuals (sex ratio = 1.26).

COLONY identified 40 parent-offspring pairs comprised of 7 mothers of the captive breeding stock and 36 captive offspring plus 4 wild offspring. ML-Relate found 45 parent-offspring pairs comprised of 27 mothers of the captive breeding stock and 33 captive offspring plus 12 wild offspring. Finally, CERVUS assigned a possible mother to each one of the offspring in the sample set, so we only considered the pairs involving a mother already found in at least one

of the other two programs as an additional support of the results. Sibship relationships were assigned prioritizing the following order: Parent-Offspring, Full-Siblings, Half-Siblings and Unrelated. The comparison of the three programs found a total of 17 mothers and 41 offspring, of which 7 individuals were wild and 34 were from the farm (Figure 3). Of the identified mothers, 12 were parents of captive offspring only, 2 were parents of wild offspring only and 3 were parents of both farm and wild offspring. Only 13.2% of farm individuals were assigned to a captive mother. Six wild individuals were full-siblings of one or more captive individuals as estimated using two programs. All Parent-Offspring and Full-Siblings relationships between a wild and a captive individual were consistent with D-loop and STR haplotypes (Supplementary table S7). A total of 90% of the wild individuals were found to be related to the farm by at least two of the three programs used, either as offspring or as sibling (Figure 4).

Population differentiation

Pairwise F_{ST} values from microsatellite data identified significant genetic differentiation between wild and two farm subsets (Founders and C1995) and also between these two subsets (Table 2). However, pairwise F_{ST} analysis based on the combination of both mtDNA markers, D-loop and STR sequences, did not show any significant differentiation.

Moreover, based on published D-loop sequence data from other wild populations of the Caribbean, South Atlantic, and Mediterranean Sea (Supplementary table S8), we found significant genetic differentiation of both wild and farm Cayman populations to all the other populations of the Atlantic and Mediterranean with two exceptions within the Caribbean (Supplementary Table S9). Dry Tortugas (DRT) is similar to the farm and wild Cayman Island

turtles and Tequesta (TEQ) is similar to wild only, but in both cases the sample size of previous studies was scarce and several haplotypes present in both farm and wild Cayman Island turtles are absent in DRT and TEQ. Moreover, we found that all Cayman Island sampling groups have high haplotype diversity when compared to the other wild populations of the Caribbean, the South Atlantic, and the Mediterranean Sea (Supplementary Figure S2). The Mixed Stock Analysis on the Foundes subset identified that the highest contributions were from Cuba (22.74%), Singer Island, Florida (USA) (14.2%), Mexico (11.76%) and Aves Island, Venezuela (11.76%) (Figure 5). This result is consistent with the known contributions to the founder stock, which are limited to 5 nesting locations and the foraging area of Nicaragua, potentially hosting individuals from all the Caribbean (Figure 1). On the other hand, only one genetic group was identified by bayesian clustering using STRUCTURE (Supplementary Figure S3).

Discussion

Biodiversity loss has become a major problem on a global scale and *ex-situ* conservation programs are a useful tool to preserve biodiversity in a wide range of taxa (Barnosky et al., 2011). It has been estimated that in the next 200 years between 4000 and 6000 species of terrestrial vertebrates will require captive breeding and reintroduction to avoid extinction (Frankham, Ballou & Briscoe, 2010). *Ex-situ* conservation actions require a scientifically informed management strategy throughout the different stages of the process, to establish self-sustained wild populations following the reintroduction. In this study, we have combined the potential of genetic analysis with background information of captive individuals across different generations, to demonstrate how the Cayman Island's reintroduction program has contributed to restore the wild population. We have also shown

how the different farm management strategies have conditioned the genetic composition of the breeding stock with added genetic value for the continuous small replacements of breeders.

Farm structure

When the CTF was founded, eggs and adults from different populations in the Caribbean Sea and the South Atlantic Ocean were taken to the farm to divide the impact of the removal of individuals among different populations (Cayman Turtle Farm, 2002). However, this strategy had an additional unexpected effect, because later studies demonstrated the profound genetic structuring among Atlantic nesting beaches (Naro-Maciel et al., 2014 and references therein). Farm haplotypes belong in fact to both lineage A and lineage B, described in Naro-Maciel et al. 2014 from the Caribbean and from the South Atlantic/Africa region respectively, consistent with the reported origin of the founder stock.

The founder stock was thus characterized by high initial diversity coupled with an expected low relatedness among the individuals. However, the breeding stock suffered a reduction in October 2001 due to Hurricane Michelle when it was reduced from 355 to 87 adult individuals, only 34 belonging to the initial founder stock. Individuals born on the farm, mainly from the cohort 1995, were kept for breeding purposes in order to increase the size of the breeding stock after the hurricane, representing now the 72.9% of the present captive breeding stock. The high percentage of F_1 breeders not assigned to any of the current founder females, (80.2 %) shows the contribution of the adult turtles lost in the hurricane or in previous management actions. Thus, F_1 breeders remain a potentially valuable source of diversity to the wild population.

The reduction of the breeding stock caused by the hurricane and the subsequent use of a large number of individuals (189) of one cohort (C1995) in the breeding stock, reduced the farm genetic variability at nuclear and mitochondrial markers. Moreover, this management strategy has increased the degree of genetic relatedness within farm individuals (Table 1) due to the higher proportion of breeders sired by the same parents when using this many individuals from a single generation. In contrast, the levels of variability of the MCF_1 group are higher, with no signals of inbreeding and lower relatedness values. This suggests that continuous small replacements of the breeding stock using individuals from different cohorts is a better strategy to maintain diversity, when possible. In any case, the loss of variability and increased relatedness are expected consequences of any captive breeding program due to genetic drift, especially those lacking genetic management (Ralls & Ballou, 1986; Witzenberger & Hochkirch, 2011). Furthermore, the higher observed (H_o) than expected (H_e) heterozygosity in C1995 and the MCF_1 fits the expected outcome when individuals from different populations reproduce (Witzenberger & Hochkirch, 2011), as their parents belong to the original founder stock. The observed variations in diversity provide valuable knowledge for future management actions in the farm, for instance, while deciding which individuals to keep for the breeding stock or as a basis for a directed reproduction program. The correct management of captive stock meant for reintroduction is a critical point for any ex-situ program, since the selection of captive breeders will reflect in the future wild reintroduced population. For this reason, the genetic balance of the captive stock has to be taken under consideration not only at the beginning, but also throughout the whole project to ensure a genetic combination as optimal as possible.

Relationship with the wild population

During the past 40 years, the CTF has been releasing hatchling and yearling turtles following the headstarting method in order to avoid the high rates of mortality during their early life stages (Bell et al., 2005). Although in the 1980s several studies declared the former wild population extinct (King, 1982; Stoddart, 1980), the Cayman Islands currently hosts a nesting population. Fifty-seven of these nesting females were captured and sampled, but ongoing tagging studies suggest that there are around 100-150 nesting females (ongoing data collection). Our sibship reconstruction showed that the farm had a significant contribution to the wild population since 90% of the wild nesting females in Grand Cayman are offspring, full- or half-siblings of female captive breeders. Consequently, most mothers and fathers (assigned or inferred by the program) of wild breeding females were either permanently captive in the farm or escaped from the captive breeding stock (Supplementary Table S10). The contribution of the farm to the wild population should be considered a minimum, since potential captive parents for the wild breeders and current wild nesters might be part of the farm breeding stock lost in 2001. Furthermore, the contribution of the younger breeders has not yet shown its impact on the population, due to marine turtles' long life cycle as the released individuals may need between 15 and 19 years to reach maturity, depending on stage of release.

Considering the large number of related individuals detected among captive and wild populations it is not surprising to find no significant differences in haplotype frequencies even for the two mitochondrial markers combined. The two groups share the highly frequent CM-A3.1_6-8-4-4 (30%), but also some rare haplotypes, such as CM-A13.1_5-7-7-4 (<5%) and CM-A27.1_5-9-4-4 (<5%), which further reinforces the relatedness between

captive and wild populations. On the contrary, microsatellites show significant differences between wild and two farm subsets, C1995 and founders, which could be due to the contribution of males to nuclear markers. For this reason, although the success of the reintroduction has already been determined by the outcome of the present analysis, the genotyping of male individuals or the reconstruction of male genotypes (Wright et al., 2012; Phillips, Mortimer, Jolliffe, Jorgensen, & Richardson, 2014) could refine the actual contribution of the farm to the wild population.

Due to the lack of historical samples of the original wild Cayman nesting population for genetic analysis, it is not possible to know the extent of the impact of the farm reintroduction program on it but our results indicate two possible scenarios. On one hand, the original wild population could have been completely replaced by captive individuals and thus the 10% of unrelated wild individuals could be some of the captive individuals lost in 2001 during the hurricane Michelle or their descendants or siblings. As evidence, the four South Atlantic exclusive haplotypes in the wild population are found in individuals related to the farm as full- or half-siblings. Therefore, these haplotypes may have been inherited from captive individuals not present in our breeding sample. In fact, the possible escape of captive individuals caused by the hurricane could be considered an accidental reintroduction. On the other hand, the few wild females with no relationship with captive turtles could be the remains of the original wild population. In fact, these non-related individuals presented haplotypes typically found in other Caribbean populations. In the context of a captive breeding or reintroduction program, these scenarios highlight the importance of collecting samples from wild individuals of a population on the edge of extinction, whenever possible. In fact, the gathering of original wild samples would facilitate

the identification of original and reintroduced individuals of the future recovered population, resulting in more accurate management decisions.

Comparison with other natural populations

Any reintroduction program is usually associated with a decrease in genetic diversity due to the reduced size of the captive stock and to the maintenance of the captive population that may lead to major problems caused by inbreeding depression (Edmands, 2007; Witzenberger & Hochkirch, 2011). Although in 1980 the number of farm founder breeders (208) doubled the optimum suggested by Witzenberger and Hochkirch (2011) to avoid inbreeding and loss of genetic diversity, the subsequent deaths in captivity and escapes as a result of the hurricane caused a drop in the number of founders, potentially increasing the risk of inbreeding depression. On the other hand, the different origins of these individuals might trigger the loss of individual fitness due to outbreeding, as a result of negative interpopulation hybridization (Edmands, 2007). Using Mixed-Stock Analysis we showed that the present founder stock still includes individuals from the North Caribbean region (Mexico, Costa Rica and Nicaragua) and the South Caribbean region (Guyana and Suriname) but the contribution of the south Atlantic region (Ascension) remains undetected (Figure 5). However we found the African haplotypes CM-A8.1 and CM-A42.1 in the C1995 subset. The haplotype CM-A8.1 is the most abundant in Ascension Island (Naro-Maciel et al., 2014), one of the source populations of the founder stock. The haplotype CM-A42.1 is exclusive from Poilao (Patricio et al., 2017) where it coexists at low frequency with CM-A8.1. As the populations of Poilao and Ascension are genetically similar (Patricio et al., 2017), finding the CM-A42.1 haplotype in the farm would imply that this haplotype is also found in Ascension Island but yet has to be discovered. Considering that after the hurricane catastrophe only 28

founder females out of 148 survived, this reduction probably resulted in a potential extensive loss of haplotypes in the founder stock but that were transmitted to the F_1 and potentially also to the reintroduced individuals (Supplementary Table S3). Therefore, the former founder stock could have presented African and south Atlantic haplotypes, now not detected in the founders, that could be found in the future in wild breeders if admixture does not compromise their fitness.

The levels of variability of mtDNA D-Loop found in captive and wild females are similar or higher when compared to other populations of green turtles from the Atlantic Ocean or the Mediterranean Sea analyzed in other studies (Figure 5). The explanation of the high diversity found in the CTF may rely on the great number and high diversity of origins of the farm breeders' founder stock. This diverse origin can be easily detected by an increase of the observed heterozygosity on the individuals that resulted from the admixture of the founders (MCF_1 and C1995); any offspring from parents of different origin are much more likely to have high levels of heterozygosity, due to the parents not sharing common alleles. However, F_{IS} values of wild Cayman females are positive and significant despite their high relatedness to the farm. The admixture of individuals from genetically differentiated units can affect the fitness and reproductive capacity of the offspring because of outbreeding depression (Weeks et al., 2011), by disrupting fine-scale local adaptation or epistatic interactions (Weber et al., 2012). Tentative evidence has been proposed for such an inbreeding-outbreeding tension in an Indian Ocean population of hawksbill turtles (Phillips, Jorgensen, Jolliffe & Richardson, 2017). In the case of the Cayman Islands, both admixed breeding farm females and sampled wild females seem to be fully capable of reproduction, suggesting that outbreeding depression is not likely to be relevant. Nonetheless, the monitoring of diversity

along with the study of the reproductive success of the wild population, as well as the farm, is extremely important, in order to evaluate any long-term impact on natural populations. Monitoring studies rarely evaluate reintroduction effects of F_2 or F_3 generations, despite some of the negative effects of outbreeding may appear in late generations (Edmands, 2007). For instance, a study on artificially translocated pink salmon has detected outbreeding depression in F_2 hybrids resulting from spatially separated populations (Gilk et al., 2004). For these reasons, when forming a founder stock for captive breeding, although the gathering of individuals from distinct genetic populations is a solid concept, the genetic composition of the populations should be previously tested to minimize the risk of outbreeding depression. Therefore, a continuous genetic monitoring of wild Cayman nesting events (including fertility and variability records) would be crucial to investigate fitness consequences after different genetic groups have mixed (Edmands 2007).

Concluding remarks

In this study, we have shown that the reintroduction program of green turtles in the Cayman Islands has greatly impacted the recovery of the wild population since 90% of the wild population is related to the turtles in the farm. This reintroduction has been fueled by a high genetic diversity due to the diverse origin of the founders used to start the captive population. Considering these results, we suggest to scientifically control the future mating of the captive breeding stock to avoid outbreeding or inbreeding in the captive population while recording fitness values of fecundity and survival. The success of the reintroduction program opens new challenges for the future management of the wild population. Further monitoring should assess whether the recovered population is self-sustainable and is essential to detect and prevent eventual negative impacts on natural populations of the

Caribbean. This monitoring is necessary because in species with long life cycles, such as green turtles, potential shifts in fitness could only be detected in the long term. In this study, we evaluated a reintroduction program 40 years after its implementation. However, the ideal scenario for any reintroduction program would be to incorporate genetic studies from the beginning. Future captive breeding programs with reintroduction purposes can benefit from following a few recommendations that arise from this study. Firstly, founder stock individuals should be collected from the genetic region of reintroduction, to avoid the mixing of unrelated genetic groups and the risk of outbreeding. Secondly, genetic pedigrees could be used to program appropriate breeding strategies to maintain genetic diversity, minimize inbreeding in the captive stock and select individuals for the reintroduction. Finally, a temporal monitoring of the wild population should be performed including information regarding its status prior the reintroduction. Scientifically informed *ex-situ* conservation actions might have higher chances of success in the recovery of endangered species.

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Data accessibility

A list of all the sampled individuals that includes the sample code, population (farm or wild), mtDNA haplotype, STR repeats and microsatellite genotypes is available as Supplementary Dataset S1.

Author contributions

BG, ACB, CC, MP, and JMB conceived and designed the study. WM sampled all of the Cayman Turtle Farm (CTF) breeders and provided data from the CTF databases. GEP, JMB, LC, and JB coordinated the sampling of the wild nesting females. AB, CM, VO, and CC did the laboratory analysis. AB, CM, CC, and MP conducted the data analysis with inputs from ACB, BG, and JMB. AB, CC, VO, and MP wrote the manuscript with input all of the authors.

Tables

Table 1. Genetic diversity values of each sample subset. The farm breeding stock was subdivided into Founders (from the original founder stock), MCF_1 (individuals of the breeding stock born at the Cayman Turtle Farm) and C1995 (individuals born in 1995 at the Cayman Turtle Farm). The table shows number of samples used with each marker (N), allelic richness (Ar), expected (H_e) and observed (H_o) heterozygosities, degree of relatedness (Rel) (values significantly higher than those obtained by random permutations are marked with $^{\#}$), inbreeding coefficient (F_{IS}) (values significant for Hardy Weinberg disequilibrium are marked with *), number of haplotypes (Haplo), haplotype diversity (H) and nucleotide diversity (π). For microsatellite values, standard errors are specified in brackets.

		FARM			WILD
		Founders	C1995	MCF_1	
Microsatellites	**N**	25	189	43	57
	Ar	8.538 (0.592)	7.644 (0.181)	7.885 (0.369)	7.821 (0.399)
	He	0.717 (0.037)	0.702 (0.034)	0.719 (0.029)	0.693 (0.042)
	Ho	0.681 (0.04)	0.72 (0.038)	0.751 (0.038)	0.664 (0.046)
	Rel	-0.021 (0.0023)	-0.003$^{\#}$ (0.0004)	-0.012 (0.0018)	-0.009 (0.0016)
	F_{IS}	0.05*	-0.025*	-0.042	0.045*
MtDNA	**N**	25	41	19	57
D-Loop	**Haplo**	8	8	7	12
	H	0.703	0.578	0.602	0.573
	π	0.0069	0.0038	0.0043	0.0039
STRs	**Haplo**	10	13	9	16
	H	0.877	0.806	0.848	0.814
D-Loop + STRs	**Haplo**	13	16	11	19
	H	0.703	0.578	0.602	0.573

Table 2. Pairwise F_{ST} values among the wild population and the different groups of the farm. Microsatellite results are shown below the diagonal and the results of the combination of D-loop and STR markers are shown above the diagonal. The values in bold are significantly different after FDR correction ($FDR_{0.05}$ = 0.020).

	Wild	Founders	C1995	MCF_1
Wild	0	-0.0075	-0.0001	-0.0114
Founders	**0.012**	0	-0.0049	-0.0156
C1995	**0.005**	**0.015**	0	-0.0035
MCF_1	0.007	0.033	-0.017	0

Figures

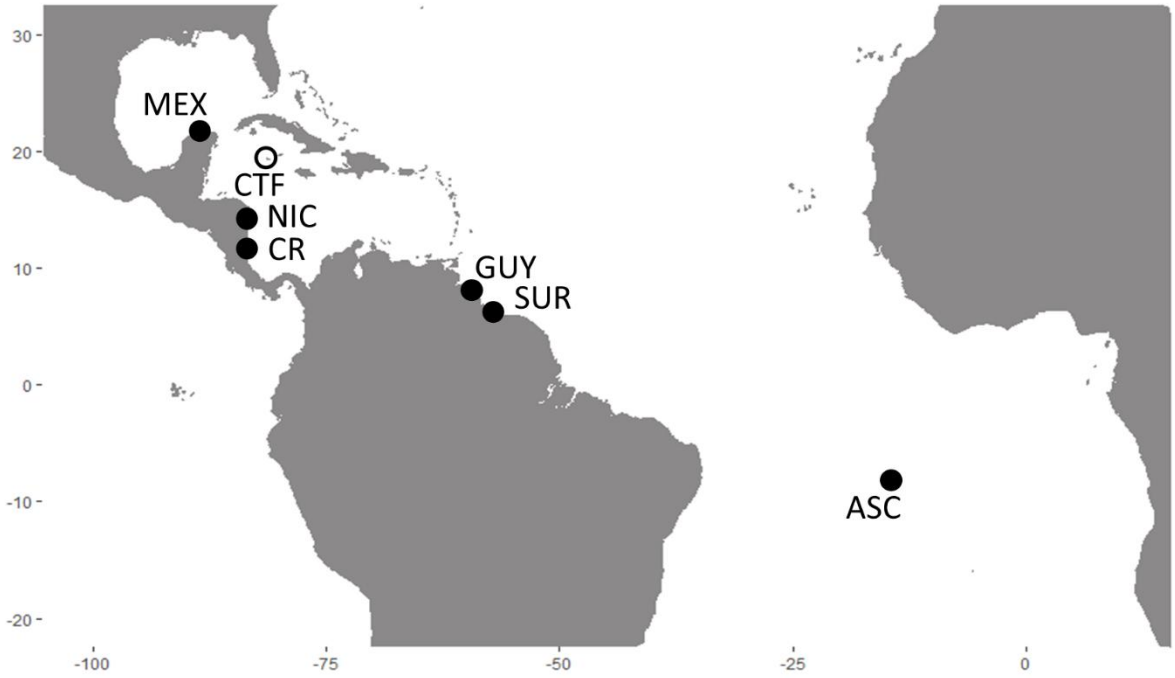

Figure 1. Map of founders of the Cayman Turtle Farm (CTF). Wild adult turtles and eggs were taken from populations in Mexico (MEX), Costa Rica (CR), Guyana (GUY), Suriname (SUR) and Ascension Island (ASC) and from the foraging area of Nicaragua (NIC) (for details on adults and eggs see Supplementary Table S1 and S2). Locations of founder's origin are marked with black circles and the Cayman Turtle Farm is marked with an empty circle.

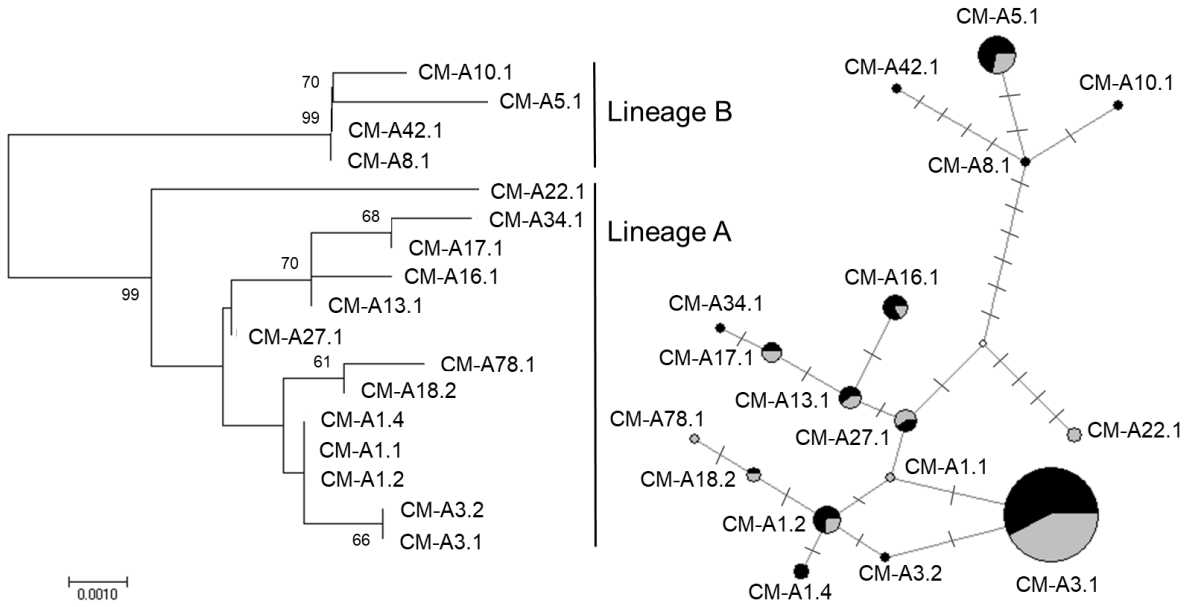

Figure 2. Genetic relationships between the haplotypes found in the farm and wild samples of the Cayman Islands. Left: Neighbor-Joining haplotype tree, middle-rooted at the longest branch, indicating maximum likelihood bootstrap values higher than 60%. Top branch represents lineage B, and bottom branch represents lineage A. The new haplotype found (CM-A78.1) belongs to lineage A. Right haplotype network of the individuals of the Cayman Islands. Connecting lines show single mutational changes between haplotypes. The red dot represents an unsampled intermediate haplotype connecting sampled haplotypes. The size of the pies represents haplotype frequencies of farm (blue) and wild (grey) individuals.

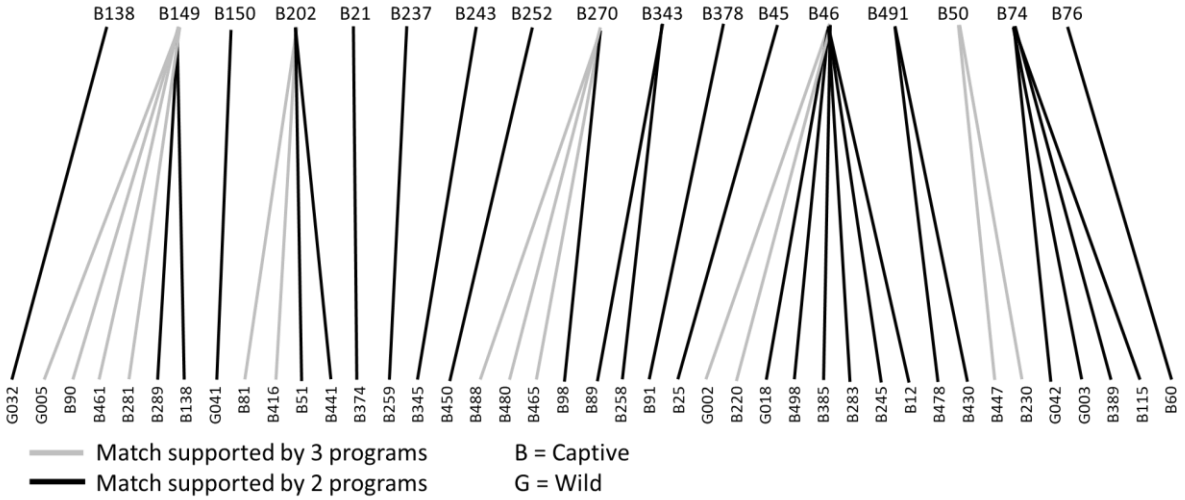

Figure 3. Pedigree of individuals of the Cayman Islands Turtle farm breeding stock. Parent-Offspring pairs were inferred by COLONY (Jones & Wang, 2010), CERVUS (Marshall et al., 1998) and ML-Relate (Kalinowski et al., 2006). The top row consists of captive individuals inferred as mothers; the bottom row consists of wild and captive individuals, which assigned to the mother from the farm. Black lines represent matches supported by all 3 programs, while grey lines represent matches supported by 2 programs.

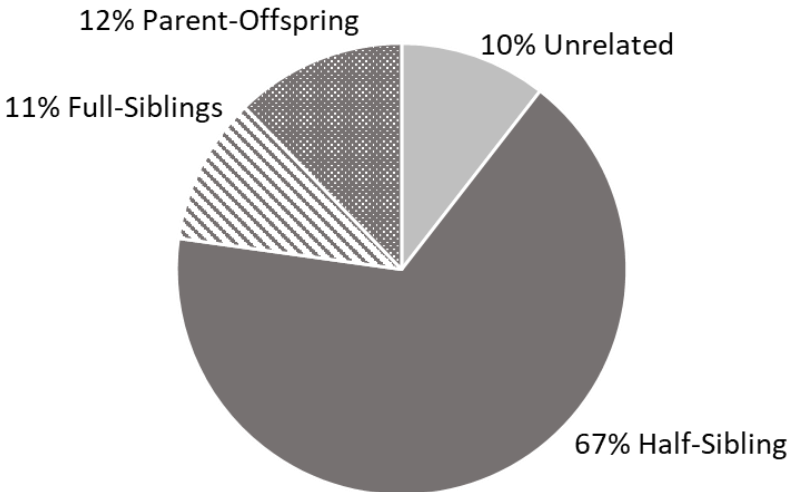

Figure 4. Parentage assignment of wild individuals to the farm breeding stock. All assignments are supported by at least 2 of the 3 programs used (COLONY (Jones & Wang, 2010), CERVUS (Marshall et al., 1998) and ML-Relate (Kalinowski et al., 2006)).

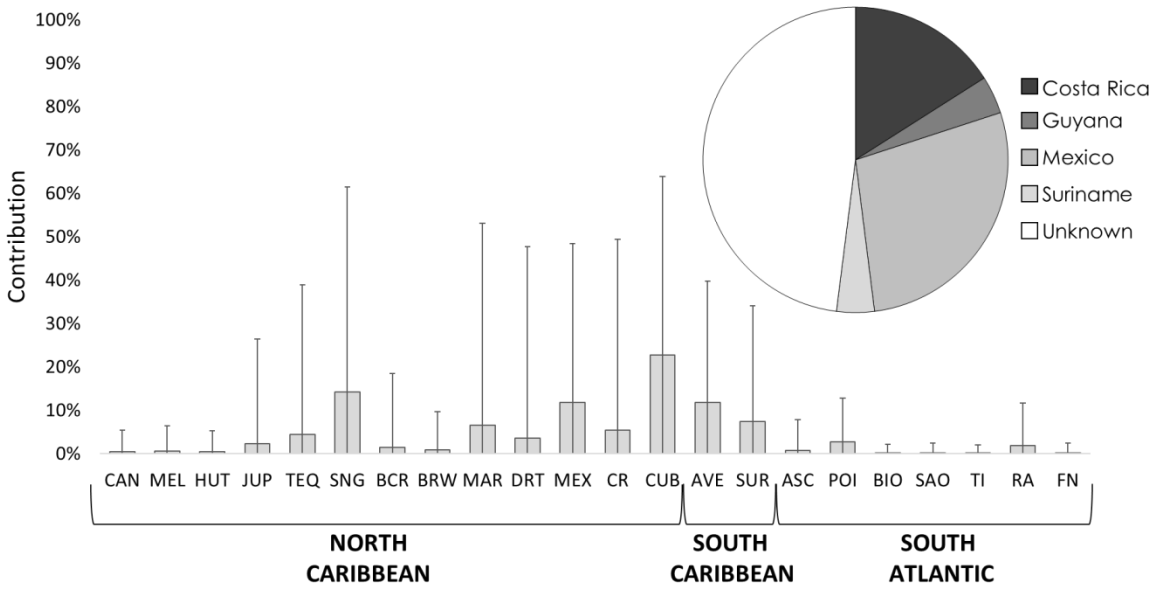

Figure 5. Mixed-Stock Analysis of the Founder subset against wild populations based on short (400bp) D-loop sequences to include samples of known origin of the founders for which long sequences were not available (i.e. Mexico). The highest contribution appears to be from Cuba (CUB), Singer Island (SNG) (Florida), Mexico (MEX) and Aves Island (AVE), corresponding to the putative geographic areas of origin of these individuals**.** The pie graph represents the origin of founder individuals as reported in the Cayman Turtle Farm background data; to the 'unknown' category belong individuals who are known to have wild origin but lack the information on the specific collection site. North Caribbean populations are CAN = Canaveral National Seashores, MEL = Melbourne Beach, Archie Carr National Wildlife Refuge, HUT = Southern Hutchinson Island, JUP = Northern Jupiter Island, TEQ = Tequesta (Southern Jupiter Island), SNG = Singer Island, BCR = Boca Raton, BRW = Hillsboro, MAR = Key West Archie Carr National Wildlife Refuge and DRT = Dry Tortugas National Park (all in Florida, USA) (Shamblin et al., 2015a), MEX = Quintana Roo (Mexico) (Encalada et al., 1996), CR = Tortuguero (Costa Rica) (Bjorndal, Bolten & Troeng, 2005) and CUB = Cuba (Ruiz-Urquiola et al., 2010). South Caribbean populations are AVE = Aves Island (Venezuela) and SUR = Matapica (Suriname) (Bolker, Okuyama, Bjorndal &Bolten, 2007). South Atlantic populations are ASC = Ascension Island, BIO = Bioko Island (Equatorial Guinea), SAO = Sao Tome (Formia, Godley, Dontaine & Bruford, 2006), TI = Trinidad Island (Trinidad y Tobago), RA = Rocas Atoll and FN = Fernando de Noronha (Brazil), and POI = Poilao (Guinea Bissau) (Shamblin et al., 2015b; Patrício et al., 2017).