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Population genetics of the endangered Knysna seahorse, *Hippocampus capensis*

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Abstract

The evolutionary history of the endangered Knysna seahorse, *Hippocampus capensis*, and the extent of gene flow among its three known populations, were investigated using 138 mitochondrial DNA control region sequences. Similarly high levels of genetic diversity were found in two of the populations (Knysna and Keurbooms Estuaries), whereas diversity in the third population (Swartvlei Estuary) was lower. Although most haplotypes are shared between at least two populations, based on the haplotype frequency distributions the three assemblages constitute distinct management units. The extant population structure of *H. capensis* suggests that the Knysna seahorse originated in the large Knysna Estuary. The presence of seahorses in the two smaller estuaries is either the result of a vicariance event at the beginning of the present interglacial period, colonization of the estuaries via the sea, or a combination of the two.

Keywords: control region, gene flow, estuaries, genetic structure, *Hippocampus capensis*, mtDNA

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Introduction

The Knysna seahorse, *Hippocampus capensis* Boulenger, 1900, was the first seahorse species to be listed as endangered on the IUCN Red Data List (Hilton-Tyler 2000). This status is derived from its limited distribution and, consequently, its low global abundance and vulnerability. The species is endemic to South Africa and is the only seahorse species known to exclusively inhabit estuaries.

Extensive SCUBA surveys in the region during 2000–2002 revealed that the current distribution of Knysna seahorses is restricted to the Knysna, Swartvlei and Keurbooms Estuaries on the south coast of the Western Cape Province, South Africa (Lockyear, Seahorse Research Group Knysna, personal communication; Fig. 1). Reports of any additional populations, e.g. in the Klein Brak Estuary (Whitfield 1995), could not be confirmed. Human settlement along all three estuaries (with the associated industrial, domestic and recreational activities) poses a severe threat to the survival of the species (Skelton 1987). Apart from human activities, the seahorses are also exposed to natural hazards. Freshwater floods regularly result in seahorse mortality (Grange, personal communication, Russell 1994) and tend to be more severe in the Swartvlei and Keurbooms Estuaries than in the larger Knysna Estuary (Lockyear, personal communication).

From a phylogeographical perspective the distribution of the estuarine Knysna seahorse is interesting. As the distribution of *H. capensis* is closely linked to suitable habitat, with most individuals being restricted to subtidal vegetation in shallow water (Lockyear, personal communication), some population genetic structuring may exist among and even within estuaries. Previous genetic studies on population structure in teleosts have dealt extensively with freshwater species (Waters & Burridge 1999; Waters *et al*. 2001), marine species (Pogson *et al*. 1995; Gold & Richardson 1998; Mamuris *et al*. 1999; Nesbø *et al*. 2000; Bowen *et al*. 2001; Planes *et al*. 2001; Planes 2002) and diadromous species (i.e. species whose life cycles include both freshwater and marine phases; Gyllensten 1985; Thomas *et al*. 1986; Ayvazian *et al*. 1994; Chenoweth *et al*. 1998), but a paucity of information is available on estuarine endemics. Therefore, our study is filling an important gap.

The fact that Knysna seahorses are not capable of powerful independent locomotion and are thus unable to actively disperse to other estuaries via the marine habitat suggests that genetic differentiation among populations may be high. However, passive dispersal by means of currents cannot be ruled out. It is unlikely that Knysna seahorses may establish themselves in the marine habitat because of a combination of low water temperatures

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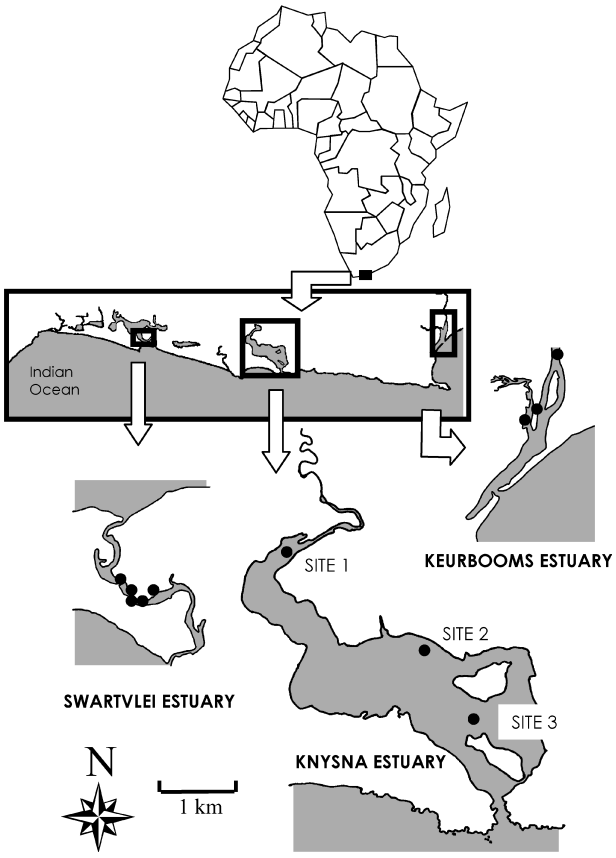

Fig. 1 Geographic localities and relative sizes of estuaries sampled in this study.

associated with occasional upwelling events (Bower & Crawford 1981; Schumann 2000), scarcity of suitable habitat (Branch & Branch 1992) and scarceness of food, as the coastal region is far less productive than estuaries (Day *et al.* 1981; Branch & Branch 1992), but a small portion of migrating seahorses may survive these adverse conditions for a sufficiently long period of time to allow them to colonize new habitats. *H. capensis* is able to survive salinities ranging from one to 59 (Riley, unpublished data, cited in Whitfield 1995), and captive individuals have reproduced successfully in seawater over several generations (Gunter, Port Nolloth Sea Farms, personal communication), supporting the notion that high salinity does not present a barrier to gene flow.

However, even if the potential for gene flow is high, unique haplotypes or differences in haplotype frequencies may exist among the three populations as a result of genetic divergence due to differences in environmental conditions among the three estuarine systems. The three estuaries inhabited by *H. capensis* differ considerably with regard to hydrological aspects. The Knysna Estuary (estuarine mouth: 34°04′ S, 23°03′ E) is by far the largest of the three estuarine systems, covering a water surface area of ≈ 19 km^2 (Geldenhuys 1979). The mouth of the estuary is characterized by a rock formation of Ordovician origin known as the Knysna heads (Toerin 1979), which maintains a large, permanently open estuary mouth. The large input of tidal marine water and the limited inflow of freshwater ensure that much of the Knysna Estuary's faunal diversity is of marine origin. The coexistence of marine, diadromous and endemic estuarine forms results in the Knysna Estuary having the highest biodiversity of any South African estuary (Grindley 1985), and the presence of at least two rare fish species (*H. capensis* and the goby *Pandaka silvana*) confers a high conservation value to this system. The mouth of the Keurbooms Estuary (34°02′ S, 23°23′ E) is located ≈ 42 km east of the Knysna Estuary. This estuary is fed by the Keurbooms and Bitou rivers, and covers an area of ≈ 2.7 km^2 (Duvenage & Morant 1984). The highest mean annual runoff estimate for the Keurbooms and Bitou Rivers has been 1.6×10^8 m^3 (Noble & Hemens 1978), which is higher than the highest value measured for the Knysna Estuary (1.3×10^8 m^3, Pitman 1981). This, in combination with a much smaller estuary area and a small inlet, suggests that the impact of freshwater floods on the Keurbooms Estuary's fauna is much more severe than in the Knysna Estuary. The Swartvlei system (34° S, 22°46′ E), which is located ≈ 26 km west of the Knysna Estuary, consists of a lower estuarine zone and an upper lake. The estuary is the smallest of the three systems with a water surface area of 2 km^2 (Liptrot 1978) and is periodically isolated from the sea by a sand bar, which forms in its mouth area due to a combination of low freshwater input and longshore winds (Whitfield *et al.* 1983). The mean annual runoff from this estuary's catchment area is comparatively low (6.6×10^7 m^3, Anonymous 1978), but mass mortalities occur when the sand bar is breached (Russell 1994; Teske personal observation).

In order to devise adequate management strategies for an endangered species, it is important to investigate its population history, geographical partitioning throughout its range, and distribution of genetic diversity (Avise 1989; O'Brien 1994). Based on conventional meristics, morphometric work and limited sequencing of the mitochondrial cytochrome *b* gene, Toeffie (2000) found that a certain amount of variation may exist between the Knysna and Swartvlei populations, and that mixing of the two populations should be avoided to preserve local adaptations until more information becomes available. In this study, mitochondrial DNA (mtDNA) control region (CR) rapidly evolving right domain sequences were used to investigate the above issues in greater detail. We were particularly interested in identifying areas of high genetic diversity, determining the level of gene flow among populations inhabiting the three estuaries (in order to assess whether they constitute distinct management units according to the definition of Moritz 1994) and to investigate whether there is any evidence of genetic substructuring within the large

Knysna Estuary. In the absence of any additional data, this mtDNA information will be important as a first step in identifying areas of high conservation value and to provide preliminary guidelines for management strategies regarding the translocation of seahorses among or within estuaries.

Materials and methods

Sample acquisition

A total of 138 specimens were sampled from all three estuaries (Fig. 1) during 2001–2002. In most cases, tissue samples were obtained nondestructively by taking small fin clips (< 1 mm^2) from the lower edge of the dorsal fin of living seahorses, and these were stored in 70% ethanol. This method has been used previously to obtain tissue samples from seahorses by Kvarnemo *et al*. (2000). Because of the high conservation status of *Hippocampus capensis*, we tested it thoroughly on seahorses bred in captivity and did not find any adverse effects (additional information is available from the authors on request). To test possible population substructure within the large Knysna Estuary, three sites were chosen in regions of the estuary which SCUBA surveys had identified as containing high densities of seahorses (Lockyear, personal communication; Fig. 1). Thirty seahorses were arbitrarily sampled from each of these three sites. Site 1 was located at the head of the estuary, and was characterized by turbid water and low salinity. Site 2 was located in the middle section of the estuary, which is characterized by higher salinities and slower current velocities (Largier *et al*. 2000). Site 3 was located in close proximity to the mouth. This portion of the estuary is characterized by near seawater salinities, cooler water temperature and strong tidal currents (Largier *et al*. 2000). Seahorses found at the two upper sites were all adults, whereas those at site 3 were all juveniles and subadults. Tissue samples from seahorses in the Swartvlei Estuary consisted partly of fin clips obtained from living seahorses (6 individuals), and partly of entire pectoral fins obtained from seahorses that had died following a breaching event (24 individuals). Samples originated from a large area in the middle section of the estuary (Fig. 1). The Keurbooms population is considered the smallest of the three assemblages (Lockyear, personal communication), and was believed to be extinct until a number of individuals were found in 2002. Hence, a permit was granted to sample only 18 individuals. These originated from sites in the upper estuary, two of which were located in the lower Bitou River, and one was located in the upper part of the Keurbooms Estuary (Fig. 1).

DNA extraction, amplification and sequencing

Genomic DNA was isolated from fin clips by proteinase K digestion followed by a standard phenol–chloroform extraction procedure (Sambrook *et al*. 1989). Samples were subsequently resuspended in 50 μL of TE buffer.

A set of primers was designed to amplify 533 nucleotides of CR right domain (forward primer: HCAL2: 5′-CACACTTTCATCGACGCTT-3′; reverse primer: HCAH2: 5′-TCTTCAGTGTTATGCTTTA-3′). This portion of the control region was chosen because it amplified most readily. Primers designed to amplify the left domain or the whole control region did not routinely amplify polymerase chain reaction (PCR) product, which was a particular problem in the case of small fin clips from juveniles and degraded DNA from dead specimens. We are confident that the gene fragment sequenced is mitochondrial rather than a nuclear pseudogene because no heterozygous sequences were identified and no multiple bands were amplified.

The DNA of 138 specimens of *H. capensis* was amplified with PCR. Each 50 μL PCR contained ≈ 1 ng/mL of total genomic DNA, 0.2 μM of each dNTP, reaction buffer including 100 mM NaCl, 0.1 mM EDTA and 20 mM Tris–HCl (pH 8.0), 0.4 μM of each primer, 2.5 mM of $MgCl_2$ and 1 unit of thermostable polymerase. The PCR profile consisted of an initial denaturation step (5 min at 94 °C), followed by 35 cycles of denaturation (30 s at 94 °C), annealing (1 min at 50 °C) and extension (1 min at 72 °C), and a final extension step (10 min at 72 °C). PCR products were directly cycle-sequenced using a BigDye sequencing kit (Applied Biosystems) and the data were analysed on a 3100 AB automated sequencer. The 5′ portion (light strand) of the fragment amplified contains a long repetitive series of up to 12 thymine nucleotides. Owing to difficulties in sequencing through this array, the fragment was sequenced in the reverse direction only. As a control, PCR amplification and subsequent sequencing reactions were repeated for every tenth sample. In all cases, the duplicate sequences were identical to the original sequences.

Data analysis

Sequences were aligned by eye in PAUP Version 4.0 beta 10 (Swofford 2002). A homologous region of 402 nucleotides was obtained for all individuals. Fifteen haplotypes were obtained, and these have been deposited in GenBank (Accession nos AY149664–AY149678). To characterize genetic variation among sampling sites and populations, estimates of nucleotide diversity (π), haplotype diversity (h) and mean number of pairwise differences (d) were obtained using the software package DNASP (Rozas & Rozas 1999). In order to minimize the effects of unequal sample sizes among populations, haplotypes of the Knysna and Swartvlei populations were subsampled. Each subsample consisted of 18 or 30 sequences randomly selected from the total set of sequences using a random number generator (http://www.randomizer.org). In order to calculate means and standard deviations of genetic indices,

nine subsamples were created in each case. An intraspecific phylogeny for *H. capensis* was inferred using the program TCS (Clement *et al*. 2000), and an analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) was conducted to investigate population structure within the Knysna Estuary and among the three estuaries. A distance matrix was constructed using the Tamura Nei model (Tamura & Nei 1993), the corresponding gamma shape distribution parameter was calculated using maximum likelihood in PAUP, and the significance of Φ-statistics was tested using 10 000 permutations of a nonparametric permutation approach described in Excoffier *et al*. (1992). F_{ST} (Wright 1965), Φ_{ST} from AMOVAS and Φ_{ST} from pairwise analyses were estimated as measures of population differentiation, using the program ARLEQUIN Version 2.000 (Schneider *et al*. 2000).

The populations were tested for evidence of recent genetic bottlenecks by implementing a graphical method introduced in Luikart *et al*. (1998). Haplotypes were assigned to frequency classes based on their particular frequency of occurrence within a population. Frequency classes were then plotted against the number of haplotypes assigned to each class. As rare alleles are more likely to become purged from a population during a bottleneck than intermediate or abundant alleles, fewer haplotypes should be found in the lowest frequency class than in one or more intermediate frequency classes. A simulation programme (ALLELOCIDE, available from the authors on request) was written to identify the size at which a randomly mating population which has undergone a recent catastrophic reduction in population size, e.g. due to a freshwater flood, is at risk of losing genetic diversity (i.e. undergoes a genetic bottleneck). In order to determine by how much the female effective population size of the Knysna population would have to decrease for this population to risk losing haplotypes, the following hypothetical model was employed. The starting population consisted of 30 000 individuals, haplotype proportions reflected those found in the Knysna population, and the number of individuals was reduced in increments of 100, each run being repeated 100 times. The starting population size was then gradually decreased to approach the value at which the first haplotypes were lost (10 000 individuals, 5000 individuals and 1000 individuals). Ten runs were then carried out with a starting population of 1000 individuals, the number of individuals being decreased in increments of 20 and each run being repeated 100 times. The percentage of replicates per run in which haplotypes were lost and the mean number of haplotypes lost in 100 replicates were recorded.

Historical demographic patterns of the Knysna population were investigated by making joint maximum likelihood estimates of the parameters θ (= $2N_f\mu$, where N_f is the effective female population size and μ is the hypothetical mutation rate for mtDNA control region right domain) and *g* (the exponential growth parameter) using the program FLUCTUATE Version 1.3 (Kuhner *et al*. 1998). In order to comply with the data requirements of this program, four rare haplotypes were removed, and two alternative methods were employed. For the first method, homoplasies were eliminated by changing nucleotides found at problematic positions to represent the same nucleotide as those found in the most closely related ancestral haplotype. To compensate for this, the changed nucleotide substitution was introduced elsewhere so that the net number of mutations remained unchanged. For the second method, the homoplasic site at position 344 was removed, resulting in a total of only seven haplotypes. In both cases, randomly created input trees were used for each run. If the program is run for sufficiently long, any bias created by the starting tree should be lost (Kuhner personal communication). Watterson's (1975) segregating sites estimate was used as the initial estimate of θ for each run, and a transition/transversion ratio of 12.9 was estimated using the maximum likelihood algorithm in PAUP. Historical N_f values were calculated using the formula $N_t = \theta\, e^{-(g\mu)t}$, where N_t is the effective female population size at time t in the past (Kuhner *et al*. 1998). The present female effective population size was determined using the formula $N_f = \theta$ generation time^{-1} μ^{-1}. A divergence time of 1 Myr per 2% sequence divergence has been widely used for bony fishes (Brown *et al*. 1979; Bermingham & Avise 1986; Grewe *et al*. 1990), but this mutation rate is based on the entire mtDNA molecule. The specific mutation rate for the noncoding control region is higher, and rates used in the recent literature range between 3.6%/Myr (Donaldson & Wilson 1999) and 5–10%/Myr (Brunner *et al*. 2001). As calibration of a molecular clock is not possible in *H. capensis*, hypothetical estimates of θ and *g* were determined using three possible mutation rates: 2, 3.6 and 5%/Myr. The generation time of seahorses is probably no greater than 1–3 years; sexual maturity is attained within 1 year (Whitfield 1995), and seahorses kept in captivity live up to at least 3 years (Lockyear 1997). Hence, results are reported using generation times of 1, 2 and 3 years. For comparison, the parameter θ was also calculated using pairwise estimations as implemented in ARLEQUIN. The parameter θ_S (Watterson 1975; Tajima 1989) is based on the number of segregating sites and the parameter θ_π (Tajima 1983) is based on the number of nucleotide differences.

Harpending's raggedness statistic (Harpending 1994) was calculated in order to determine whether there was significant genetic evidence for population growth or stasis using ARLEQUIN. Departure from selective neutrality was tested using Fu's F_s, Fu and Li's D^*, and Fu and Li's F^* (Fu & Li 1993; Fu 1997). Fu's F_s is particularly suited to detect departures from neutrality in nonrecombining sequences characterized by a high frequency of rare haplotypes and recent mutations (Fu 1997). If a significant departure from selective neutrality is detected only when

Table 1 Frequency of occurrence of *Hippocampus capensis* CR right domain haplotypes in the Knysna Estuary (three sampling sites with 30 individuals each), Keurbooms Estuary (18 individuals) and Swartvlei Estuary (30 individuals). Segregating sites of derived haplotypes are compared with nucleotides at corresponding sites in haplotype 1 (in bold). Nucleotides identical to the ones in haplotype 1 are marked with a dot. The position of each segregating site is indicated by a three-digit number

	n					Segregating sites									
	Knysna sites					0	0	1	1	1	1	2	2	3	3
						0	2	0	3	8	8	4	9	4	9
Haplotype	1	2	3	Keurbooms	Swartvlei	9	2	5	1	7	9	5	4	4	5
1	**15**	**13**	**8**	**3**	**9**	**T**	**C**	**A**	**T**	**C**	**—**	**A**	**C**	**T**	**G**
2	0	1	0	1	0	·	·	·	·	·	C	·	·	·	·
3	1	1	5	1	0	A	·	·	·	·	·	·	·	·	·
4	1	0	0	0	0	·	·	·	·	T	·	·	·	·	·
5	1	3	4	0	0	·	·	·	·	·	·	·	·	C	·
6	0	0	1	0	0	·	·	·	·	·	C	·	·	C	·
7	0	0	1	1	0	·	·	·	·	·	·	·	T	C	·
8	4	5	3	1	0	·	T	·	·	·	·	·	·	·	·
9	2	1	0	0	0	·	T	·	·	·	·	·	·	C	·
10	0	0	1	0	0	·	T	·	·	·	·	G	·	C	·
11	0	0	0	1	0	·	T	·	·	·	·	·	T	C	·
12	4	6	7	9	0	·	T	G	·	·	·	·	·	·	·
13	0	0	0	0	1	·	T	G	·	·	·	·	·	C	·
14	1	0	0	0	0	·	T	G	·	·	·	·	·	C	A
15	1	0	0	1	20	·	T	G	C	·	C	·	·	·	·

implementing Fu and Li's tests, this suggests that background selection is the more likely cause of this deviation from neutrality. If, however, only F_s is significant, departure from the assumption of neutrality is more likely due to population growth or hitchhiking (Fu 1997). Fu's F_s was calculated using ARLEQUIN with 10 000 simulated samples, whereas Fu and Li's tests were determined using DNASP.

Results

The segment of the mitochondrial control region sequenced in 138 individuals of *Hippocampus capensis* contained 10 variable sites (1 indel, 8 transitions and 1 transversion; Table 1). These polymorphic sites defined 15 haplotypes, of which 6 were unique to the Knysna population, 1 was unique to the Keurbooms population and 1 was found exclusively in the Swartvlei population. Six haplotypes were represented by single individuals. Although the sampling area in the Swartvlei Estuary was considerably larger than each individual sampling site within the Knysna Estuary, the number of haplotypes found was lower than at any one of the three sites within the Knysna Estuary. Haplotype 1 was the most abundant haplotype at each of the sites within the Knysna Estuary, and the second most abundant in the Keurbooms and Swartvlei populations. Haplotype diversity and expansion coefficients were similar in the Knysna and Keurbooms estuaries, and lower in the Swartvlei Estuary, whereas nucleotide diversity was higher in the two smaller estuaries than in the Knysna Estuary because their haplotypes tended to be more divergent (Table 2). Magnitudes of haplotype and nucleotide diversities were not drastically affected by subsampling. This suggests that smaller sample sizes were sufficient to calculate good approximations of the genetic indices of each of the three populations.

No significant structure was found among the three sites within the Knysna Estuary using AMOVA (% variation among populations = 0; $\Phi_{ST} = 0$; $P = 0.8$). Hence, the sequences from this population were combined in subsequent analyses. An AMOVA revealed significant structure between the three estuarine populations (% variation among populations = 29.49; $\Phi_{ST} = 0.331$; $P < 0.01$), and pairwise comparisons found significant structure among all three estuaries (Table 3).

A star-like pattern was identified in the haplotype network constructed using the program TCS (Fig. 2), which indicates recent ancestral monomorphism followed by a population expansion (Slatkin & Hudson 1991). Using some of the criteria outlined in Crandall & Templeton (1993), haplotype 1 has been designated as ancestral. It is the most abundant haplotype in the Knysna Estuary, and it has the most pivotal position in the network. Owing to several equally parsimonious solutions, the relationship between haplotype 1, and particularly the most derived haplotypes, is ambiguous. The relationships between such haplotypes were resolved using the criterion suggested by

Table 2 Genetic diversity indices and sample sizes (*n*) of the Knysna, Swartvlei and Keurbooms populations, as well as individual sampling sites within the Knysna Estuary. Indices include number of haplotypes (H); haplotype diversity (*h*); nucleotide diversity (π), number of polymorphic (segregating) sites (*S*); mean number of pairwise nucleotide differences (*d*); expansion coefficient (*S*/*d*)

Site	*n*	H	*h*	π	*S*	*d*	*S*/*d*
Knysna combined	90	13	0.78	0.00353	10	1.41	7.1
Knysna combined*	30	7.22 ± 1.20	0.77 ± 0.04	0.0032 ± 0.0004	5.9 ± 1.2	1.19 ± 0.37	5.9 ± 3.9
Knysna							
1	30	9	0.73	0.00348	8	1.40	5.7
2	30	7	0.76	0.00299	5	1.20	4.2
3	30	8	0.84	0.00425	7	1.71	4.1
Swartvlei	30	3	0.48	0.00461	5	1.86	2.7
Knysna combined*	18	5.78 ± 1.30	0.75 ± 0.08	0.0032 ± 0.0006	4.9 ± 1.3	1.27 ± 0.23	3.8 ± 0.6
Swartvlei*	18	2.55 ± 0.53	0.46 ± 0.05	0.0045 ± 0.0005	4.5 ± 0.5	1.80 ± 0.20	2.9 ± 0.5
Keurbooms	18	8	0.75	0.00458	7	1.84	3.8

*Values represent mean (± SD) calculated from nine subsamples. Each subsample consisted of a number of sequences randomly chosen from the original sample using a random number generator (http://www.randomizer.org).

Table 3 Pairwise comparisons of genetic structure among the three estuaries; below diagonal: F_{ST} values; above diagonal: Φ_{ST} values

	Knysna	Keurbooms	Swartvlei
Knysna		0.102**	0.432**
Keurbooms	0.075*		0.257**
Swartvlei	0.253**	0.343**	

*P = 0.05; **P = 0.01.

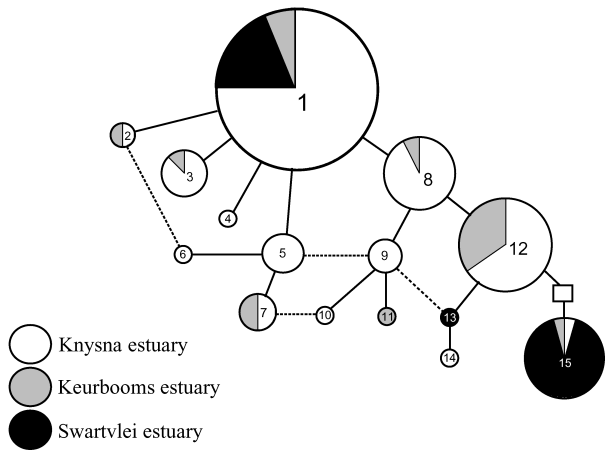

Fig. 2 Haplotype network of CR rapidly evolving right domain haplotypes of *Hippocampus capensis*. Each haplotype is represented by a circle, the size of which indicates the frequency at which it was found. The square represents an internal node haplotype not present in the sample. Each line represents a single nucleotide substitution. Proportional representation of haplotypes in the different estuaries is indicated by subdivision of circles into up to three sections. All connections shown have a probability of ≥ 95% of being true, but connections represented by solid lines are more highly supported than those represented by broken lines by virtue of criteria outlined in the text.

Crandall & Templeton (1993): whenever a derived haplotype was linked to two older haplotypes by an equal number of mutational steps, the derived haplotype was connected to the older haplotype that was present in the population at greater frequency than the other.

The large number of rare haplotypes in the Knysna population resulted in an L-shaped distribution of haplotype frequencies (Fig. 3a), which suggests that this population has not experienced a recent genetic bottleneck (Luikart *et al*. 1998). In contrast, the presence of a single rare haplotype in the Swartvlei Estuary is indicative of a recent bottleneck or founder event in this population (Fig. 3b). A second plot of haplotypes found in the Knysna Estuary based on 9 sets of 18 sequences each (Fig. 3c), shows that the L-shaped pattern is still recovered when using smaller sampling sizes. The Keurbooms population was also characterized by a high frequency of rare haplotypes (Fig. 3d), but haplotype 1 was the second most frequently encountered haplotype in this population. Using the program ALLELOCIDE, a reduction in population size to ≈ 500 individuals resulted in the first rare haplotype/s being lost from the data set of Knysna haplotypes. The number of runs in which haplotypes were lost then increased rapidly as population size was reduced further (Fig. 4). A mean number of one haplotype was lost at a population size of ≈ 150 individuals. Using a different initial population size of 30 000, 10 000 or 5000 individuals had no effect on the results.

The population history of *H. capensis* was investigated using the Knysna population only. Apart from providing the largest data set and containing most of the haplotypes found, this population is most likely to be the most important from a conservation perspective (see Discussion). As the AMOVA results show a lack of subpopulation structure within the Knysna Estuary, the following tests were performed for a pooled data set of this population. A large number of short (1000 steps each) and long (10 000 steps

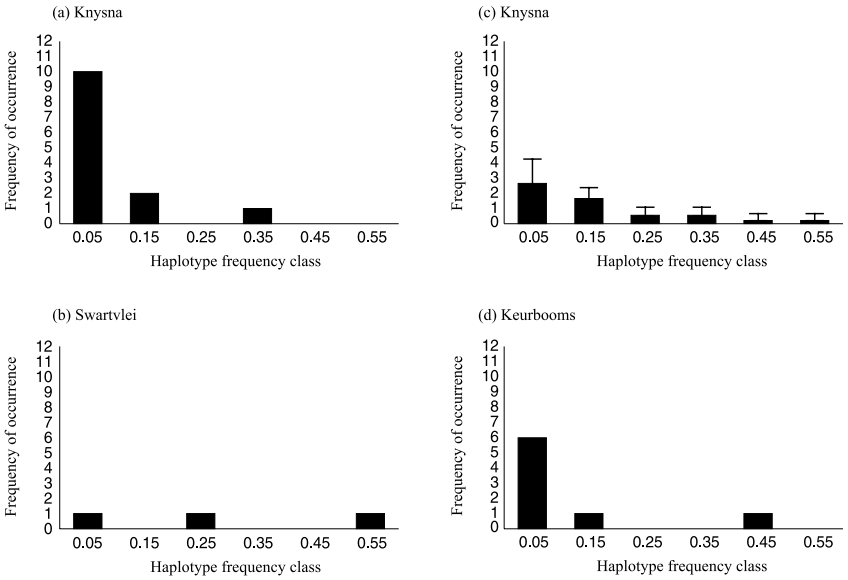

Fig. 3 Control region right domain haplotype frequency distribution; (a) Knysna population (90 individuals); (b) Swartvlei population (30 individuals); (c) Knysna population (18 individuals resampled nine times from original 90; bars indicate means, whiskers represent positive standard deviation); (d) Keurbooms population (18 individuals).

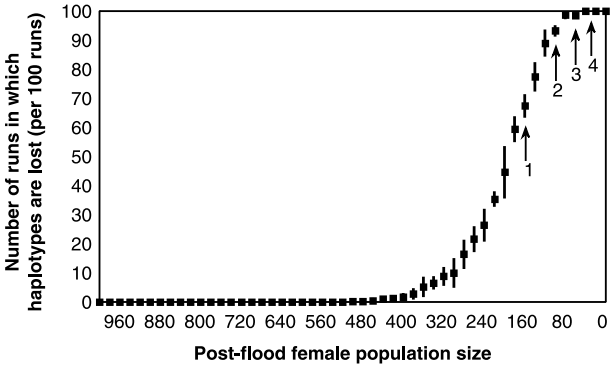

Fig. 4 Loss of haplotypes as a result of a catastrophic reduction in population size. Results plotted from Visual Basic simulation program ALLELOCIDE; starting population size: 1000; increments: 20 individuals; number of replicates: 100. Values plotted are means (± SD) from 10 repetitions. Numbers below some data points represent the mean number of haplotypes lost in 100 replicates of a particular run.

each) chains was run for both methods in the program FLUCTUATE, and sampling increments of 20 were used for both short and long chains. As results differed slightly depending on the number of chains run, the number of chains was increased from 15 to 40 short chains in increments of 5, and approximately half the number of long chains in each run. We report θ and g as the means of six runs. The θ-value (± SD) calculated using the first method was 0.130 ± 0.021 with a simultaneously estimated exponential growth parameter (g) of 1399 ± 152. The results for the second method were as follows: θ (± SD) = 0.210 ± 0.124 and g (± SD) = 1999 ± 768. Values of θ, calculated using approaches based on pairwise comparisions, were $\theta_S = 0.0049 \pm 0.0019$ and $\theta_\pi = 0.0037 \pm 0.0025$.

The recent population expansion suggested by the star-like phylogeny and the large growth rates was confirmed using the raggedness statistic, which did not reject the sudden expansion model ($r = 0.05$, $P = 0.41$). A negative and significant Fu's F_s test for the combined data set from the Knysna population further supports the evidence for a demographic expansion of this population ($F_s = -7.34$, $P < 0.01$). Fu and Li's D^* and F^* for the Knysna population, however, were not significant ($D^* = -2.08$, $P > 0.05$, $F^* = -1.91$, $P > 0.05$). This excludes the possibility that background selection is responsible for the departure from neutrality, and justifies the application of the coalescent-based ML algorithm used in FLUCTUATE to investigate population history. Estimates of θ and g obtained using the first method were used to generate plots of historical population sizes over time (Fig. 5). Assuming that population growth was relatively constant, the approximate age of the Knysna population is defined as the point in time at which the historical N_f value is < 1% of the estimated present effective female population size. Calculated using the first method, this lies between 65 000 and 486 000 years ago, whereas results using the second method placed the age of the population between 46 000 and 339 000 years (not shown).

Discussion

Genetic structure and gene flow

The samples from the Keurbooms and Swartvlei estuaries each contained a single private haplotype (haplotypes 11 and 13), whereas the Knysna Estuary contained a total of six (Table 1). The remaining seven haplotypes were shared among at least two estuaries. Although it is possible that

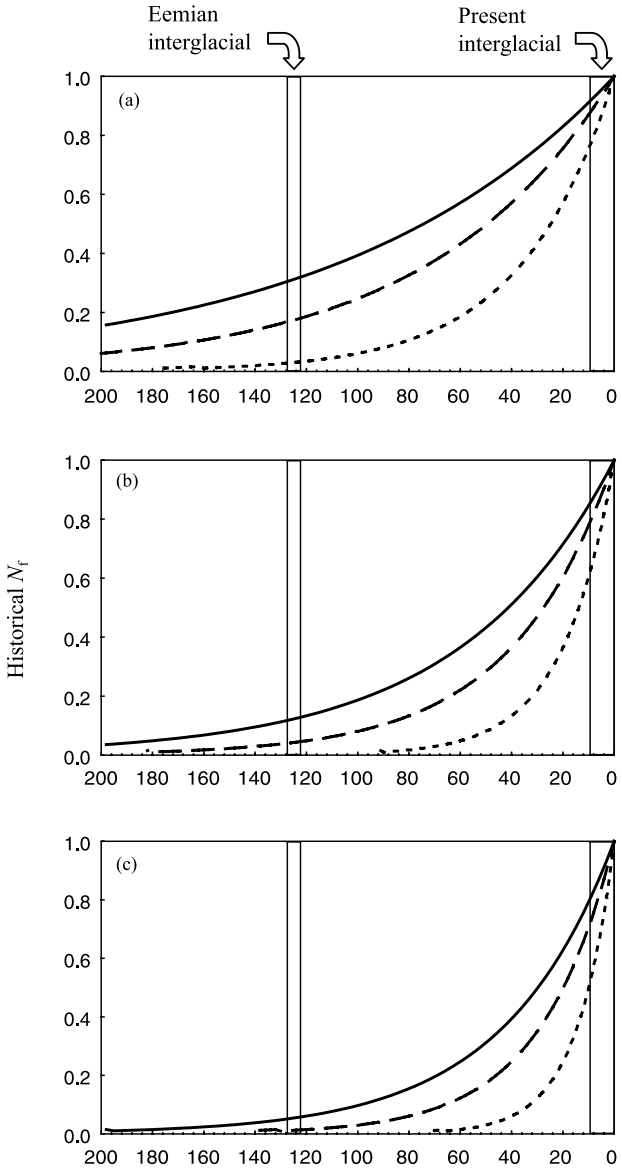

Fig. 5 Plots of historical effective female population sizes (N_f) at three putative CR right domain mutation rates: (a) 2%/Myr; (b) 3.6%/Myr; (c) 5%/Myr. Three possible generation times are plotted: 1 year (dotted line), 2 years (broken line) and 3 years (solid line). Historical N_f values are expressed as proportions of present N_f.

due to sampling efforts not all of the unique haplotypes present in the three populations were represented in the data set, it is interesting to note that clear haplotypic frequency differences exist among the estuaries. The significant structure (supported by both F_{ST} and Φ_{ST} values, Table 3) found among the three estuaries suggests that each of the populations constitutes a distinct management unit *sensu* Moritz (1994). However, the conclusion that the populations in the three estuaries may be evolving relatively independently under different stochastic processes, is at best tentative, because it is based on low sample sizes and a single neutral marker only. The same can be said about the conclusion that the population structure in the Swartvlei population differs considerably from that detected in the other two populations because this population experienced a recent population bottleneck, founder event or loss of haplotypes due to random genetic drift due to small population size: although the right domain of the control region provides good resolution at the demographic level, Luikart *et al*. (1998) recommended that analyses for genetic bottlenecks should involve 5–20 independent loci. We find no evidence for population substructure within the Knysna Estuary and these analyses were based on a total sample size of 90 individuals, suggesting that this conclusion is fairly robust. Lack of genetic structure in the Knysna Estuary is also supported by the fact that site 3 was inhabited exclusively by juveniles and young, nonbreeding adults, whereas the two upper sites were inhabited by adults. Juveniles and adults may live spatially separated in preferred habitat types. The most apparent explanation for this pattern is the nature of the vegetation in the different areas. Site 3 is characterized by very dense patches of seagrass, which seem to be an ideal habitat for smaller seahorses, whereas larger seahorses would find it difficult to move within them. The more open vegetation in the estuary's upper reaches, however, may be more suitable for large seahorses.

Evolutionary history of Hippocampus capensis

Although it is difficult to date the exact time when *Hippocampus capensis* diverged from its marine ancestors because the parameters estimated with FLUCTUATE are imprecise due to an uncertain genealogy and biased because they are based on only one locus (Kuhner *et al*. 1998), the results of this study can be used as a rough indication to date such an event. Using two approaches to determine haplotype relationships, three different mutation rates and three different generation times, the age of the Knysna population has been estimated to between 46 000 and 486 000 years (late Pleistocene). Environmental conditions along the coast during the Pleistocene differed considerably from present-day conditions, and several factors suggest that it was unlikely that tropical or subtropical marine seahorses that may have given rise to *H. capensis* were able to reach the Knysna Estuary during this time. At present, the coastal waters of the east coast are dominated by the warm Agulhas Current, which aids in the southwards dispersal of tropical marine organisms (Heydorn 1978; Blaber 1981; Turpie *et al*. 2000). This dispersal occurs mainly during the austral summer, when sea surface temperatures are in the region of 24 °C and the Agulhas Current is more defined than during winter (Heydorn 1978), suggesting that it is facilitated both by water temperature

and current strength. During the Pleistocene, sea-surface temperatures along South Africa's east coast were up to 4 °C cooler and the Agulhas Current was considerably weaker (Lindesay 1998). Moreover, although the Agulhas Current is deflected away from the coast in the Eastern Cape region because the continental shelf widens, transport of tropical species towards the south coast is possible as eddies may transport water from the Agulhas Current towards the shoreline (Branch & Branch 1992). In contrast, during much of the Pleistocene, the Agulhas Current was deflected eastwards just south of Madagascar (Lindesay 1998). However, a founder event is conceivable during the Eemian interglacial period (127 000–122 000 years ago), a short warm phase within the Pleistocene during which sea surface temperatures along the south coast were higher than present-day temperatures (southwest coast: +3.8 °C; southeast coast: +0.9 °C; Crowley & North 1991). The fossil record indicates that much of the south coast's Eemian coastal marine fauna included species currently restricted to warmer Indian Ocean currents off the Eastern Cape Province, KwaZulu Natal Province, or even Mozambique (Martin 1962; Davies 1971). This suggests that the tropical or subtropical marine seahorses that gave rise to the Knysna population may also have been considerably more abundant in the region during the Eemian interglacial than they are today. On the basis of the results of the first method to calculate θ and g, a generation time of 1–2 years and a mutation rate of 3.6%/Myr or slightly higher, the possibility of a founder event during the Eemian interglacial is well supported (Fig. 5b). The age of the population suggests that apart from being geographically isolated from its sister species and living in a habitat that is likely to be inhospitable to other seahorses because of unstable physical and chemical conditions, *H. capensis* may also be phylogenetically distinct, and the high conservation status of this species thus seems justified. Preliminary results based on control region sequences of closely related marine seahorses confirm this (Teske *et al.* unpublished data).

As there is no suitable habitat along the south coast of South Africa, and *H. capensis* is highly specialized to survive in estuaries, physiological adaptation to the estuarine environment must have taken place in one of the three estuaries. If one assumes that a founder event took place during the Eemian interglacial, then environmental and biotic conditions characterizing the three estuaries must have been similar to present conditions, as sea surface temperatures were similar, and the sea level was only 4 m higher than it is today (Gribnitz & Kent 1989). The Knysna Estuary, with its high haplotypic diversity, seems the most likely location for this event to have taken place. First, the marine-dominated lower reaches of this estuary provide an optimal transition zone for the gradual adaptation of marine species to the estuarine environment. Second, the large size of the estuary reduces the detrimental effects of freshwater floods and can also support a larger population of seahorses. This improves the prospect for an estuarine population to establish itself and ensures its long-term survival. Third, because of its permanently open mouth, the estuary is readily accessible to seahorses migrating along the coastline. The notion that the Knysna population is the oldest is also supported by the fact that a rare estuarine goby, *Pandaka silvana*, is endemic to this estuary (Penrith & Penrith 1972). In contrast, no endemics have been found in the other two estuaries. The evolutionary history of *P. silvana* may be similar to that of *H. capensis*, because like the majority of seahorses, all other species in the genus *Pandaka* are exclusively tropical and marine.

Convergence of the Knysna, Keurbooms and Swartvlei rivers lower on the continental shelf during the colder period between the Eemian and present interglacial, and possibly a vicariance event brought about by a subsequent rise in sea levels, may eventually have resulted in three extant populations. The lower genetic diversity of the population residing in the Swartvlei Estuary can be explained by subsequent loss of haplotypes due to genetic bottlenecks. However, this scenario does not explain why the population in the Keurbooms Estuary has a significantly higher genetic diversity. Preliminary survey data suggest that seahorse densities are approximately equal in the Keurbooms and Swartvlei estuaries (Lockyear, personal communication), and the small sizes of these two estuaries suggest that their populations are thus likely to be similar in size and considerably smaller than the Knysna population. An additional explanation for the observed haplotype pattern is the small-scale migration of seahorses between the estuaries. Assuming that the source population resides in the Knysna Estuary, it is more likely that seahorses that have been flushed out of this system end up in the Keurbooms Estuary rather than the Swartvlei Estuary, because the prevailing coastal current flows eastwards (Branch & Branch 1992). The fact that site 3 in the Knysna Estuary was inhabited exclusively by juveniles and subadults, and that no population substructure was found within this estuary, suggests that juveniles and adults may be spatially separated. Dispersal within the estuary is probably accomplished passively through tidal currents, and it is likely that some juvenile seahorses may be flushed out to the sea before finding suitable habitat. This may provide the two smaller estuaries with an infrequent but continuous input of new colonists.

Although the Keurbooms population is characterized by high genetic diversity, the different ratio of haplotypes to that in the Knysna population (and particularly the low abundance of the common haplotype 1), suggests that this population may nevertheless undergo fluctuations in population size. The estuary experiences floods of substantial magnitude (Duvenage & Morant 1984), which are likely to

be more detrimental to the fauna than floods of similar magnitude in the Knysna Estuary, on account of the Keurbooms Estuary's smaller size. For that reason, the Keurbooms population may be dependent on gene flow from the Knysna population in order to maintain its high genetic diversity. Lastly, it cannot be ruled out that presence of seahorses in the two smaller estuaries is the result of recent introductions by humans, as suggested by Kok (1981). This is particularly plausible in the case of the Swartvlei population: although anecdotal evidence suggests that the species has been present in the Swartvlei Estuary for several decades, this system was not included in the list of estuaries inhabited by *H. capensis* in 1986 (Dawson 1986).

Population size and conservation implications

The high number of rare haplotypes in the Knysna assemblage suggests that this population is sufficiently large to tolerate floods without being at risk of undergoing a genetic bottleneck. This notion is supported by the preliminary results of survey work. The census population size of adult seahorses has been estimated to be ≈ 60 000 (Lockyear & Teske unpublished data). The population has an even sex ratio and > 90% of the males were found to be pregnant during the breeding season (Lockyear & Teske personal observation). This suggests that the effective female population size (N_f) may be close to 30 000. Behavioural work on the Australian seahorse *H. whitei* has shown that these form long-term faithful pair bonds (Vincent & Sadler 1995). Although it is not known whether this is also the case in *H. capensis*, the fact that male seahorses do not collect eggs from more than one female at a time (Vincent 1995), suggests that there is no justification for incorporating reproductive skew into calculations of N_f.

Methods to calculate effective population sizes based on genetic data yield higher values than the survey results (in all cases $\mu = 0.036$/Myr and generation time = 1.5 years were used to calculate N_f from θ, and standard deviations are given). Approaches based on pairwise comparisons of the number of segregating sites or the number of nucleotide differences, arrived at values approximately two to three times as high as N_f calculated from census estimates (N_f [from θ_S] = 90 740 ± 35 185; N_f [from θ_π] = 68 519 ± 46 296). Even higher values of N_f were calculated using FLUCTUATE. When jointly estimating θ and g, effective female population sizes of $2.4 \times 10^6 \pm 0.4 \times 10^6$ (first method) and $3.8 \times 10^6 \pm 1.2 \times 10^6$ (second method) were determined. The considerable differences between the estimates of N_f calculated using different methods suggests that these should be interpreted cautiously because of uncertainties about input variables and the use of a single neutral marker. Given the caveat that the survey prediction is accurate, the fact that N_f values calculated using genetic methods were higher may be an indication that the population size of *H. capensis* in the Knysna Estuary has decreased considerably during the species' short evolutionary history. However, even a drastic decline in population numbers is unlikely to be detected using the genetic methods employed here, because it is unlikely to result in a loss of rare haplotypes. Unless severe and for a prolonged period (which is not applicable in the case of a freshwater flood), it is also unlikely to have an effect on the proportional abundance of each haplotype within the population. The results of the simulation program ALLELOCIDE illustrate this point. A 1% chance of losing the first rare haplotype in the Knysna population (i.e. haplotype/s lost in 1 of 100 runs, Fig. 5) exists when population numbers have been reduced to 500 individuals, and the number of runs in which haplotypes are lost then increases rapidly as population size is reduced further. If we assume a present effective female population size of 30 000 individuals, then the removal of up to 98% of adult females would not result in the loss of any haplotypes. This conclusion is, however, based on the premise that all of the rare haplotypes present in the population were represented in the sample, which is highly unlikely. The actual minimum number of females remaining in the population in order for haplotypes to be lost may thus be slightly above 500 individuals. Although one also needs to consider that an Allee effect (Allee 1938) resulting from the low population density is likely to further increase the population size at which haplotypes are lost, the fact that adult seahorses were often found in aggregations (see Introduction) suggests that many of the individuals that have survived a freshwater flood may be confined to relatively small areas.

The difference between theoretical and observed effective female population sizes may be an indication that anthropogenic pressures during the past decades could already have had a significant negative impact on the Knysna population. The present rate at which construction developments and other human activities are increasing along the estuary is all the more alarming in the light of these findings. The resulting habitat degradation may make recovery of the population after a naturally occurring disaster such as a freshwater flood increasingly difficult. Whichever scenario resulted in the observed mtDNA population structure in the two smaller estuaries (i.e. vicariance followed by possible genetic bottlenecks in the Swartvlei Estuary vs. migration resulting in recent founder events, or a combination of the two), from a ecological perspective the Knysna Estuary has the greatest potential to ensure long-term survival of the species on account of the large size of the population and the less detrimental effects of freshwater floods. In addition, our mtDNA data also indicated high mtDNA diversity in this estuary and it is thus imperative that conservation efforts for this population be prioritized. Although the three populations of

H. capensis constitute individual management units, this conclusion was reached mainly on the basis of differences in haplotype frequencies among the populations rather than a large proportion of private haplotypes. It cannot be ruled out that these differences in haplotype frequencies may be an artefact of small sample sizes or unbalanced cohort sampling in the two smaller estuaries. Because of the absence of distinct monophyletic clades of haplotypes unique to individual populations and the generally good support for the migration hypothesis, there is at this stage little reason to discourage the translocation of seahorses among the different estuaries, but only if this should become necessary. The lack of population structure within the Knysna Estuary suggests that areas temporarily affected by habitat degradation due to construction developments in the vicinity are likely to become repopulated on their own once they have reverted to their original state. Translocations in the Knysna Estuary are feasible in cases in which the degradation of formerly populated areas is expected or documented, in which case seahorses could be collected and released in more pristine parts of the estuary.

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This conservation genetic study on the Knysna seahorse reflects one of the research focuses of the evolutionary genomics group at Stellenbosch University, South Africa. The present paper is a component of P.R. Teske's Ph.D. study on seahorse population genetics and molecular systematics. P.R. Teske is primarily interested in the ecology and population structure of estuarine fishes and invertebrates, in particular estuarine endemics. M.I. Cherry has a keen interest in mating systems, and most of his work has focused on sexual selection and on brood parasitism. C.A. Matthee is mainly interested in molecular systematics and evolutionary population genetics.
