Validating the use of colouration patterns for individual recognition in the worm pipefish using a novel set of microsatellite markers

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Abstract

In studies of behaviour, ecology and evolution, identification of individual organisms can be an invaluable tool, capable of unravelling otherwise cryptic information regarding group structure, movement patterns, population size and mating strategies. The use of natural markings is arguably the least invasive method for identification. However, to be truly useful natural markings must be sufficiently variable to allow for unique identification, while being stable enough to permit long-term studies. Non-invasive marking techniques are especially important in fishes of the Family Syngnathidae (pipefishes, seahorses and seadragons), as many of these taxa are of conservation concern or used extensively in studies of sexual selection. Here, we assessed the reliability of natural markings as a character for individual identification in a wild population of Nerophis lumbriciformis by comparing results from natural markings to individual genetic assignments based on eight novel microsatellite loci. We also established a minimally invasive method based on epithelial cell swabbing to sample DNA. All pipefish used in the validation of natural markings, independently of sex or time between recaptures, were individually recognized through facial colouration patterns. Their identities were verified by the observation of the same multilocus genotype at every sampling event for each individual that was identified on the basis of natural markings. Successful recaptures of previously swabbed pipefish indicated that this process probably did not induce an elevated rate of mortality. Also, the recapture of newly pregnant males showed that swabbing did not affect reproductive behaviour.

Keywords: colouration, individual identification, microsatellites, non-invasive sampling, skin swabs

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Introduction

Techniques for the identification of individual organisms have played an important role in research in ecology, evolution and behaviour. For instance, some of the most striking breakthroughs made by von Frisch (1946), who would ultimately share the Nobel Prize for his discoveries concerning the organization and elicitation of individual and social behaviour, were facilitated by his ability to identify individual honeybees by painting them with small dots of coloured paint. In the decades following Frisch, the use of artificial markings grew in popularity as an approach for individual identification in behavioural studies, but artificial marking techniques are not entirely free of shortcomings. For example, not all species are amenable to marking and some marks simply do not last long enough to be useful. However, many taxa possess natural markings that can be used for individual identification (Würsig & Jefferson 1990), thus minimizing impacts on individual behaviour and welfare due to direct manipulation. One of the most iconic examples of the use of natural markings for individual recognition involves the identification of cetaceans through fin morphology and colouration (e.g. Katona & Whitehead 1981). Related studies, involving numerous other taxa,
have resulted in breakthroughs that would not have been possible without the use of natural markings for individual recognition (Mammals: Anderson et al. 2007; Auger-Methe & Whitehead 2007; Fish: Aoyama 2000; Kitchen-Wheeler 2010; Martin-Smith 2011; Monteiro et al. 2005; Reptiles: Schofield et al. 2008). An exhaustive compilation of the relevant bibliography on marking and tagging of aquatic animals, especially fish, was conducted by Emery & Wydoski (1987).

Fishes of the Family Syngnathidae (pipefishes, seahorses and seadragons) have become important models for studies of behavioural ecology and conservation biology (Mobley et al. 2011; Rosenqvist & Berglund 2011; Vincent et al. 2011), and both of these enterprises would benefit from methods for individual recognition. Natural markings have already proven to be useful for the identification of individuals in seadragons, Phyllopteryx taeniatus (Martin-Smith 2011) and Phycodurus eques (Connolly et al. 2002), as well as in the pipefish Nerophis lumbriciformis (Monteiro et al. 2005). Some seahorse species, such as Hippocampus erectus (Bergert & Wainwright 1997), also possess intricate facial markings, making them potential targets for the use of this type of methodology. The use of non-invasive marking techniques is especially important in syngnathid fishes as many taxa in this group are in critical need of basic life history data (Whitfield 1995; Dias et al. 2002; Morgan & Panes 2008) or used as models in studies focused on sexual selection and mating behaviour (e.g. Jones & Avise 1997; Berglund et al. 2006; Silva et al. 2009). While some studies have already successfully marked syngnathids with artificial tags, such as necklaces, elastomer implants or electronic tags (see Caldwell et al. 2011), these techniques have the potential to disrupt behaviour or increase mortality and may not be possible for some species. Therefore, it seems reasonable to use natural markings whenever possible, especially considering the low invasiveness of such an approach. Although natural markings have undeniable potential, they also have limitations, and their use for individual recognition depends primarily on two main sets of conditions: (i) markings need to be sufficiently polymorphic to allow objective differentiation even between similar individuals (Anderson et al. 2007) and (ii) markings need to be stable during an extended time period, or at least constant during the extent of a particular study. Even though the benefits of individual identification based on natural markings will usually outweigh the disadvantages, this technique should be rigorously verified for each focal species before it is put into widespread use.

Here, we assessed the reliability of natural markings as a character for individual identification in the worm pipefish Nerophis lumbriciformis based on unambiguous individual genetic assignments using eight newly described microsatellite loci. We successfully established a method based on gentle swabbing of epithelial cells along the fish’s body surface to sample DNA in a minimally invasive fashion and compared the results based on our genetic markers to those obtained from comparisons of facial markings on field-captured individual worm pipefish.

Material and methods

Sampling
Pipefish were sampled in the rocky intertidal of Viana do Castelo (41°41’58.85”N, 8°51’21.60”W) in northern Portugal. Sampling was performed monthly during spring tides (from September 2011 to September 2012) near the low water mark by inspecting the undersides of boulders where this pipefish typically occurs (Monteiro et al. 2002). Following Monteiro et al. (2005), each fish was immediately photographed for individual identification (macrodigital photographs using a Pentax Optio WG-1). Facial markings in N. lumbriciformis are asymmetric, so we photographed both sides of each individual’s head to facilitate identification in cases where the colouration patterns on one side of the head were insufficient to objectively distinguish similar individuals of the same sex and approximate size. A full-body photograph was also taken in order to gather additional phenotypic information (e.g. sex and size).

Prior to the release of each fish to the spot where it was initially captured, we applied a marginally invasive but non-destructive method of sampling DNA from epithelial cells (similar to techniques described in Le Vin et al. 2011; Martin-Galvez et al. 2011; Prunier et al. 2012). Buccal swabs (Isohelix SK2) were used to smoothly swab the fish skin in the abdominal and tail area, thus avoiding the head (in order to prevent eye or opercular damage) and the genital papilla (thus avoiding faecal contamination). All the samples were submerged in 96% ethanol and transferred to the laboratory for storage at −20 °C.

Facial pattern comparison was based on visual inspection of the photographs. In order to identify a recapture event, we used information on sex and length and compared facial markings with photographs from previous sampling events (see examples of N. lumbriciformis facial pigmentation patterns in Fig. 1). A total of six males and seven females, which had been successfully recaptured based on the photographic evidence, were selected for genotyping to validate genetically that they had indeed been recaptured. These 13 individuals were chosen to (i) include representatives of both sexes, as males and females differ in the amount of facial pigmentation (Monteiro et al. 2005) and (ii) include recapture events that were temporally distant from the
first sampling (up to 7 months) in order to confirm previous observations, indicating that facial patterns are stable across time (Monteiro et al. 2005).

**Microsatellite development**

Size-selected fragments from genomic DNA were enriched for microsatellite sequences using magnetic streptavidin beads and biotin-labelled CT, GT, GTAT and GATA repeat oligonucleotides. The enriched library was analysed on a Roche 454 platform using the GS FLX titanium reagents. The total 34,720 reads had an average length of 146 base pairs. Of these, 2,059 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible for 443 microsatellites, of which 27 were tested for polymorphism, and eight finally selected for pipefish genotyping.

**DNA extraction and amplification**

Genomic DNA was extracted from swabs of 34 distinct individuals (20 males and 14 females) for the characterization of the novel microsatellites. These samples did not include the validation of recapture events. In the case of recaptures, we extracted DNA from the swabs obtained during the first capture as well as the swabs obtained during a later recapture event. The microsatellite genotypes obtained during recaptures were used to validate the visual identification process, but were not included in the estimation of allele frequencies.

A commercial kit (Genomed JETquick) was used to extract DNA from the epithelial cells captured in each swab, following the manufacturer’s instructions. Samples were amplified for eight microsatellites (Nerlum_05025, Nerlum_16858, Nerlum_02383, Nerlum_07690, Nerlum_00750, Nerlum_06490, Nerlum_12901 and Nerlum_00629). All amplifications were performed in a 10 μL reaction volume containing 1 μL DNA, 1 μL (10X) PCR reaction buffer (Invitrogen), 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.8 μM primer set, 0.8 μM fluorescently labelled tail (FAM) (Schuelke 2000) and 0.1 U Platinum Taq DNA polymerase (Invitrogen). The PCR thermal profile used to amplify all the microsatellites began with an initial denaturation at 95 °C for 15 min, which was followed by 30 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 45 s. The fluorescently labelled tail was incorporated during eight additional cycles (95 °C for 30 s, 53 °C for 45 s and 72 °C for 45 s), which were followed by a final extension step at 72 °C for 30 min. All PCRs were conducted on a Bio-Rad MyCycler™ Thermal Cycler.

**Microsatellite analysis**

Fragment analysis was performed on an ABI 3100 capillary sequencer (Applied Biosystems) using LIZ 75-450 as the size standard (Applied Biosystems). Peak Scanner Software v1.0 (Applied Biosystems) was used to score fragment size. GenAlEx v6.5 (Peakall & Smouse 2012) was used to assess allelic diversity, allelic frequency, observed (Hobs) and expected (Hexp) heterozygosity and probability of identity (P(ID)), which reports the probability of two randomly sampled individuals from our data set having the same genotype. We tested for departures from Hardy–Weinberg equilibrium proportions (HWE) and genotypic linkage disequilibrium using Genepop (Raymond & Rousset 1995; Rousset 2008) and estimated null allele frequencies with Cervus 3.0 (Kalinowski et al. 2007). To verify that recaptured individuals, based on visual assessment of facial markings, were indeed real recaptures, we compared the genotypes obtained from the first capture to those from later putative recaptures of the same individual. We used Microchecker to compare multilocus genotypes (Van Oosterhout et al. 2004).

**Results**

The selected method for DNA sampling, based on the gentle swabbing of the outer surface of the pipefish,
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proved to be a successful alternative to other more invasive techniques, such as fin clipping. All of the samples contained good-quality DNA, with no apparent signs of degradation, suitable for microsatellite genotyping. The swabbing process did not seem to produce any negative effects in the sampled pipefish as (i) we continued to recapture individuals showing normal behaviour with no apparent scars or infections (i.e. the DNA sampling process did not seem to increase mortality risk) and (ii) some of the recaptured males became pregnant shortly after our sampling, so they remained reproductively active and attractive to females.

All the studied loci proved to be polymorphic, showing between 7 and 19 alleles per locus in the focal population. Observed heterozygosity (Hobs) ranged from 0.706 to 0.853, while expected heterozygosity (Hexp) ranged from 0.690 to 0.857 (Table 1). None of the loci displayed a significant deviation from HW expectations (Table 1), and there was no evidence for significant linkage disequilibrium among the tested markers (only one of 28 pairwise tests was significant at x = 0.05; data not shown). Consequently, estimated null allele frequencies were very low (Table 1). The P(ID) for all loci combined was extremely low (9.0 \times 10^{-11}). As a quality control mechanism, we repeated 15% of our PCR amplifications and found no changes in the observed genotypes.

All of the 13 pipefish used to test the validity of natural markings, regardless of sex or interval of time between recaptures, were easily recognizable through facial colouration patterns. In every case, these individuals with the same patterns of facial markings (see two examples in Fig. 1) collected at different sampling times also showed identical multilocus genotypes, clearly indicating that they were indeed the same pipefish.

Discussion

The worm pipefish displays a considerable number of spots, predominantly in the facial area, which form intricate patterns. Monteiro et al. (2005) recognized that these extremely polymorphic patterns, acting much like a human fingerprint, could be used for individual recognition and, after a year of aquarium observations, decided that they were sufficiently stable for use in a natural population. This inexpensive and apparently precise methodology made it possible to demonstrate that the worm pipefish is not a permanent intertidal resident, but displays strong homing behaviour when it is present in the intertidal. Interestingly, one male was recaptured in virtually the same spot within the intertidal more than a year after its initial sighting (Monteiro et al. 2005). Moreover, a total of eleven pipefish (six males and five females) were recaptured in two consecutive breeding seasons (Monteiro et al. 2006), implying that facial markings tend to be stable across long time periods. However, an unequivocal validation of the facial-marking recognition process was never formally conducted. Here, we showed that facial markings are indeed sufficiently polymorphic to permit confident individual recognition, even for males, which typically bear less intricate patterns of pigmentation than females. It should be stressed, however, that the individual recognition technique we used is not entirely non-invasive, as it still depends on fish handling for photographic records. Even so, it is probably one of the least invasive alternatives available if we aim to gather information at the individual level.

To assess the reliability of pigmentation patterns for individual recognition, we developed a novel suite of microsatellite markers, which should be useful to address other questions related to the ecology and behaviour of this species. We also tested a relatively non-invasive DNA sampling technique. The swabbing technique we used appeared to be minimally invasive and could be performed quickly in concert with the photographs we took of each pipefish. As already verified for other taxa, namely birds (Martin-Galvez et al. 2011; Yannic et al. 2011) and amphibians (Prunier et al. 2012), swabbing for DNA proved to be an extremely useful technique in worm pipefish. Most of the caveats associated with the quest for high-quality DNA, especially in fish (from behaviour to phenotype alterations), are elegantly described by Le Vin et al. (2011). In N. lumbriciformis, the use of body mucus swabbing provided enough good-quality DNA for the genotyping process without causing any observed infection or interfering with reproduction. In fact, the percentage of recaptures (number of recapture events divided by the number of captured adult individuals) obtained in this study (nearly 23%) was similar to that obtained 10 years ago (nearly 21%) when the same population was also monitored but without the use of swabs (Monteiro et al. 2005). Moreover, the frequent recapture of pregnant males indicated that swabbing did not affect normal reproductive behaviour in these individuals.

Although the primary use of the newly described microsatellites in this study was to validate the effectiveness of facial pigmentation patterns in individual identification, these two methodologies are far from mutually exclusive. In fact, both methodologies can, whenever possible, be used side-by-side, with obvious economical advantages. For instance, the use of natural markings, if properly validated, can be used for individual recognition, allowing for the monitoring of a group of individuals with minimal disturbance. The more expensive genetic fingerprinting can then be reserved for occasions...
Table 1 Summary data for eight microsatellite loci developed for *Nerophis lumbriciformis*

<table>
<thead>
<tr>
<th>Locus (accession no)</th>
<th>Repeat type</th>
<th>Primer sequence (5′-3′)</th>
<th>Ta (°C)</th>
<th>n</th>
<th>A</th>
<th>Size range (bp)</th>
<th>$H_{(Exp)}$</th>
<th>$H_{(Obs)}$</th>
<th>HWE</th>
<th>$P_{(ID)}$</th>
<th>Null allele freq.</th>
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<tbody>
<tr>
<td>Nerlum_05025 (KF317541)</td>
<td>(AGAT)$_7$</td>
<td>F: FAM-GCATAGCTCCACTTTGCTTG R: GAACTTTGGTCCGCTTGGAGG</td>
<td>56</td>
<td>34</td>
<td>9</td>
<td>128 – 164</td>
<td>0.690</td>
<td>0.765</td>
<td>NS</td>
<td>$1.4 \times 10^{-1}$</td>
<td>$-6.90 \times 10^{-2}$</td>
</tr>
<tr>
<td>Nerlum_16858 (KF317542)</td>
<td>(ATAC)$_6$</td>
<td>F: FAM-TTGGTTTGTGACGCCACATC R: ACTGATATTGGTGAGTCTGTATG</td>
<td>56</td>
<td>34</td>
<td>14</td>
<td>82 – 162</td>
<td>0.845</td>
<td>0.765</td>
<td>NS</td>
<td>$3.9 \times 10^{-2}$</td>
<td>$5.24 \times 10^{-2}$</td>
</tr>
<tr>
<td>Nerlum_02383 (KF317543)</td>
<td>(GT)$_9$</td>
<td>F: FAM-GGTGCTTCAAAATGTGTC R: GCAGTACACACATACACCTC</td>
<td>56</td>
<td>34</td>
<td>19</td>
<td>105 – 155</td>
<td>0.814</td>
<td>0.853</td>
<td>NS</td>
<td>$4.5 \times 10^{-2}$</td>
<td>$-2.36 \times 10^{-2}$</td>
</tr>
<tr>
<td>Nerlum_07690 (KF317544)</td>
<td>(AC)$_2$</td>
<td>F: FAM-TTGAACAGTAGAGAAAGCCAG R: GGCAAGCTGAGGCTTTAGGG</td>
<td>56</td>
<td>34</td>
<td>12</td>
<td>78 – 106</td>
<td>0.825</td>
<td>0.837</td>
<td>NS</td>
<td>$5.1 \times 10^{-2}$</td>
<td>$2.32 \times 10^{-2}$</td>
</tr>
<tr>
<td>Nerlum_00750 (KF317545)</td>
<td>(CAA)$_3$</td>
<td>F: FAM-TCAGTATGATGCCAACC CAC R: TCCCACATGATCTGGAACAC</td>
<td>56</td>
<td>34</td>
<td>11</td>
<td>162 – 195</td>
<td>0.779</td>
<td>0.765</td>
<td>NS</td>
<td>$7.4 \times 10^{-2}$</td>
<td>$1.14 \times 10^{-2}$</td>
</tr>
<tr>
<td>Nerlum_04900 (KF317546)</td>
<td>(AC)$_8$</td>
<td>F: FAM-GTCTGGCAACAATCCCATCAG R: CGTATGCCCAGTGACATTC</td>
<td>56</td>
<td>34</td>
<td>7</td>
<td>150 – 186</td>
<td>0.857</td>
<td>0.794</td>
<td>NS</td>
<td>$3.6 \times 10^{-2}$</td>
<td>$3.75 \times 10^{-2}$</td>
</tr>
<tr>
<td>Nerlum_12901 (KF317547)</td>
<td>(GT)$_3$</td>
<td>F: FAM-AAGGGAAGCCAAATGCAAGCG CAG R: ACGGAAGCGCTAAAGAAGCTC</td>
<td>56</td>
<td>34</td>
<td>11</td>
<td>160 – 187</td>
<td>0.843</td>
<td>0.853</td>
<td>NS</td>
<td>$4.1 \times 10^{-2}$</td>
<td>$-4.30 \times 10^{-3}$</td>
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<tr>
<td>Nerlum_00629 (KF317548)</td>
<td>(CAA)$_7$</td>
<td>F: FAM-GTGAGCTGACAGGCAACATC GC R: GTCAACTCTGCCGGAATCGG</td>
<td>56</td>
<td>34</td>
<td>12</td>
<td>149 – 191</td>
<td>0.793</td>
<td>0.706</td>
<td>NS</td>
<td>$6.8 \times 10^{-2}$</td>
<td>$4.68 \times 10^{-2}$</td>
</tr>
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</table>

Ta (°C), Temperature of annealing; n, number of genotyped individuals; A, number of alleles; $H_{(Exp)}$, Expected heterozygosity; $H_{(Obs)}$, Observed heterozygosity; HWE, Hardy–Weinberg equilibrium proportions; NS, no significant departure from HWE.
where an individual cannot easily be recognized on the basis of its natural markings (e.g. juveniles). Likewise, given the effectiveness of skin swabs, it seems sensible to suggest that, whenever feasible and especially for endangered species, fin clipping or any other more invasive DNA collection methodology could be replaced by this less invasive technique.

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N.M. was responsible for experimental design and funding. M.C. and R.M.S. carried out the fieldwork and performed the technical work. N.M., M.C., R.M.S., A.A., M.N.V. and A.G.J. analysed the data and wrote the manuscript.

Data Accessibility