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Using integrative taxonomy to distinguish cryptic halfbeak species and interpret distribution patterns, fisheries landings, and speciation

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ABSTRACT

Context. Species classification disputes can be resolved using integrative taxonomy, which involves the use of both phenotypic and genetic information to determine species boundaries. **Aims.** Our aim was to clarify species boundaries of two commercially important cryptic species of halfbeak (Hemiramphidae), whose distributions overlap in south-eastern Australia, and assist fisheries management. **Methods.** We applied an integrative taxonomic approach to clarify species boundaries and assist fisheries management. **Key results.** Mitochondrial DNA and morphological data exhibited significant differences between the two species. The low level of mitochondrial DNA divergence, coupled with the lack of difference in the nuclear DNA, suggests that these species diverged relatively recently (*c.* 500 000 years ago) when compared with other species within the *Hyporhamphus* genus (>2.4 million years ago). Genetic differences between the species were accompanied by differences in modal gill raker counts, mean upper-jaw and preorbital length, and otolith shape. **Conclusions.** On the basis of these genetic and morphological differences, as well as the lack of morphological intergradation between species along the overlapping boundaries of their geographical distributions, we propose that *Hyporhamphus australis* and *Hyporhamphus melanochir* remain valid species. **Implications.** This study has illustrated the need for an integrative taxonomic approach when assessing species boundaries and has provided a methodological framework for studying other cryptic fish species in a management context.

Keywords: biogeography, diversity, fish, fisheries, garfish, genetics, otoliths, speciation.

Introduction

Systematics forms the backbone of many ecological fields, which allows policymakers to build conservation practices around species boundaries and identify endangered populations (Haig *et al.* 2006). Despite the importance of species classification, the existence of competing species concepts complicates the separation of similar species. Many widely accepted species concepts use competing diagnostic criteria that may establish different definitions of species boundaries and can result in contradictory species diagnoses (De Queiroz 2007). These conflicting opinions can result in taxonomic inflation, and surveys have shown that studies using the phylogenetic species concept hold a 48% higher species count than those using non-phylogenetic species concepts (Agapow *et al.* 2004). Problems with determining species boundaries are especially important for cryptic species, whose morphological forms are almost indistinguishable. Delimiting cryptic species boundaries is important because similar-looking species with different life-history traits may require different conservation or management tactics (Bickford *et al.* 2007). Cryptic fish species are common in marine ecosystems where their distributions can overlap (Knowlton 2000; Takahashi *et al.* 2020), and the management implications of cryptic species discrimination are crucial for commercially exploited marine fishes, as stock management practices may affect species in different ways.

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The unified species concept defines a species simply as a 'separately evolving metapopulation lineage' and uses multiple diagnostic criteria to delimit species boundaries (De Queiroz 2007). By accepting the many different characteristics a lineage can acquire along the course of divergence as different lines of evidence to assess a species status, the unified species concept provides a solution to the discrepancies in cryptic species delimitation through integrative taxonomy (De Queiroz 2007, 2011). Integrative taxonomy aims to determine units of life from multiple complementary perspectives rather than relying on traditional morphological or more recent phylogenetic methods of species discrimination alone (Dayrat 2005). Integrative methods, combining both morphological and molecular traits, are increasingly being used to resolve the taxonomy of cryptic fish species (Delrieu-Trottin *et al.* 2022; Liggins *et al.* 2022). Examining the differences between cryptic fish species using multiple lines of evidence not only aids in resolving species concept disputes but can also confirm stock subdivision and connectivity from multiple standpoints (Izzo *et al.* 2017), thus ensuring that management practices are tailored to species-specific needs. For example, the cryptic species diagnosis of two commercially targeted wobbegong species in eastern Australia, namely *Orectolobus ornatus* and *Orectolobus halei* (Huveneers 2006), has allowed species-specific information regarding differences in distribution, reproduction, age, growth, and genetic structure to be incorporated into fisheries management (Corrigan *et al.* 2008, 2016; Huveneers *et al.* 2013).

Recent advances in genetic and morphological species-discrimination techniques, such as DNA barcoding and otolith shape analysis, can aid in resolving cryptic fish-species boundaries. DNA barcoding usually involves a comparison of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene between different species, with the number of base-pair differences between *COI* sequences acting as an indication of the level of genetic separation between different species (Hebert *et al.* 2003). Indeed, this is possible because of the utility of the 'barcoding gap', where interspecific sequence variability for congeneric *COI* sequences is almost always greater than is intraspecific sequence variability (Meyer and Paulay 2005). Although *COI* barcoding can provide a clear means of cryptic-species discrimination when morphological evidence is sparse, the use of genetic evidence alone to confirm cryptic-species status without thorough taxonomic assessment has been criticised (Moritz and Cicero 2004) and barcoding is more widely accepted when used in conjunction with other lines of genetic, morphological, behavioural, or ecological data (Padial *et al.* 2010; DeSalle and Goldstein 2019). Otolith shape analysis, involving the comparison of inner-ear bone morphology, is well established as a stock discrimination technique to compare different populations within a single species group (Stransky 2014), and has become another tool employed alongside genetic and morphological data to better distinguish cryptic fish species (Nielsen *et al.* 2010; Zhuang *et al.* 2015).

Cryptic species are common within the globally distributed garfish (or 'halfbeak') genus *Hyporhamphus*. Of the 40 species within the *Hyporhamphus* genus, many can be distinguished only on the basis of slight differences in body shape or number of gill rakers (Supplementary Table S1). The small size of gill raker structures in *Hyporhamphus* species makes them difficult to count in the field, which poses problems for the discrimination of commercially exploited species and could hinder the implementation of species-specific management practices. One pair of commercially important cryptic *Hyporhamphus* species that face this diagnostic dilemma includes the Australian-endemic garfish *Hyporhamphus australis* (Steindachner) and *Hyporhamphus melanochir* (Valenciennes). At present, gill raker count is the only diagnostic criterion for delimiting them as unique species (Collette 1974). This makes it difficult for recreational and commercial fisheries to distinguish between *H. australis* and *H. melanochir* in southern New South Wales (NSW) waters where their distribution is thought to overlap (Collette 1974). Species-level classification is important for stock management, given documented life-history differences between the two species (Supplementary Table S2). *Hyporhamphus australis* is a smaller but faster-growing species that reaches maturity at a smaller size and younger age than *H. melanochir* (Jones *et al.* 2002; Stewart *et al.* 2005). Current minimum mesh-size restrictions for ocean garfish lampara nets in NSW protect *H. australis* juveniles below 20.1-cm fork length (FL) (Stewart *et al.* 2004); however, these management practices are not designed to protect immature *H. melanochir* stocks that may also be found in NSW waters.

Whereas the level of genetic differentiation between *H. australis* and *H. melanochir* has not been thoroughly explored (but see Noell *et al.* 2001 for brief notes on mitochondrial DNA or mtDNA comparison), newer methods of discrimination such as DNA barcoding and otolith shape analysis could clarify their species boundaries. Using a nuclear DNA marker alongside *COI* barcoding can further confirm the extent of their genetic separation and, if diagnostic base pair differences are present, identify hybrid individuals (Pavan-Kumar *et al.* 2016). Hybridisation near the town of Eden (37°S, southern NSW), where the distributions of *H. australis* and *H. melanochir* are likely to overlap, has been hypothesised from the collection of a single specimen with intermediate morphological traits (Collette 1974). However, further analysis of the levels of gene exchange between the species is necessary to support this assumption.

This study aims to explore genetic and morphological differences between *H. melanochir* and *H. australis* to determine the best method of discrimination between the two species and confirm their overlapping distributions in southern NSW. Using whole fish and tissue samples collected throughout the mainland Australian distributions of both species, we assessed differences in the body morphology

and otolith shape between the two species alongside genetic differences in the mtDNA *COI* and nuclear *TMO-4C4* regions. We hypothesise that this integrated taxonomic study will identify new genetic and morphological methods to better discriminate between *H. australis* and *H. melanochir*, and that an examination of the *COI* and *TMO-4C4* regions between the species may indicate the presence of hybrid individuals.

Materials and methods

Sample collection and preparation

In total, 120 whole fish specimens were obtained from recreational and commercial catches across the mainland Australian distribution of *H. australis* and *H. melanochir* (Fig. 1, Table 1, Supplementary Table S3). All specimens were stored frozen at −20°C prior to analysis. An additional set of tissue and otolith samples was acquired from 187 fish (Table 1, Supplementary Table S4) collected by NSW Department of Primary Industry (NSW DPI) as part of their port-monitoring program between January and July of 2019. These samples were chosen to ensure a balanced mix of individuals from different age, sex, and size classes from each catch. All whole fish were processed at the Australian Museum (detailed methods and voucher numbers are outlined in the 'Supplemental detailed sample preparation' section in the Supplementary material).

All fish samples were obtained from the NSW DPI commercial-fishery port-monitoring program. The program is exempt from requiring Animal Care and Ethics Approval as all fish sampled have been captured and retained for sale by licenced commercial fishers working under approved fishing practices.

Genetic analysis – mitochondrial *COI*

DNA was extracted from a total of 307 tissue samples by using the ISOLATE II Genomic DNA Kit (Meridian Bioscience, Cincinnati, OH, USA) following the manufacturer's protocol.

A 609-base pair (bp) segment of the mtDNA *COI* gene was amplified with a combination of Fish F1/F2 and Fish R2 primers (Supplementary Table S5) following polymerase chain reaction (PCR) conditions from Ward *et al.* (2005). All mtDNA amplification by PCR, gel visualisation, and Sanger sequencing were performed by the Ramaciotti Centre for Genomics at the University of New South Wales in Sydney, Australia. The DNA sequences were aligned, edited, and trimmed to the same length by using Geneious Prime (ver. 2020.2.4, see https://www.geneious.com) and deposited in the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database (GenBank accession numbers MZ575768–MZ576070).

To determine a species ID for each fish and divide samples into species groups for downstream analysis, the trimmed and edited *COI* sequences were compared with reference

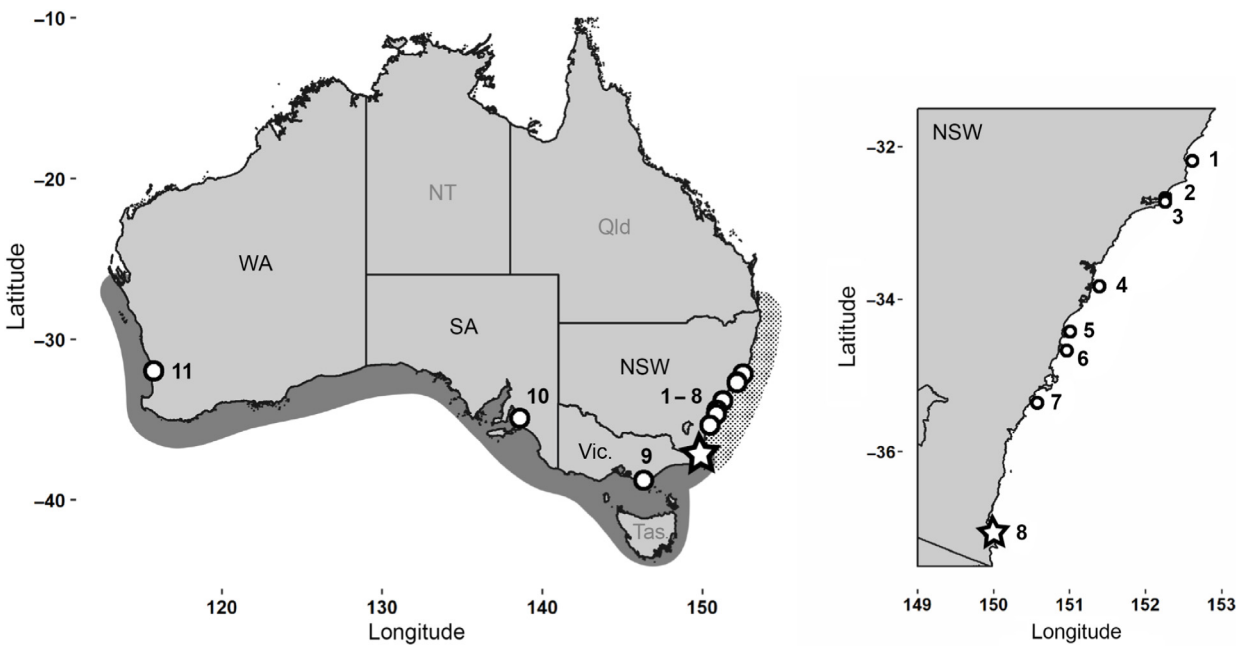

Fig. 1. Distribution and sampling locations of *Hyporhamphus australis* (distribution dotted) and *Hyporhamphus melanochir* (distribution dark grey), with New South Wales Sites 1–8 enlarged. Open circles indicate sampling locations (Eden, the town within the 56-km stretch of coastline where both species distributions overlap, is indicated with a star). Site numbers correspond with Table 1. Data on species distribution were sourced from the CSIRO CAAB Australian National Fish Expert Distributions (CSIRO 2009*a*, 2009*b*).

Table 1. Summary of sample collection date and type for each location.

Site number	Location	Sampling period	Latitude and longitude	*n* (tissue and otolith)	*n* (whole fish)
1	Forster, NSW	Mar. 2019–Feb. 2020	32.18°S, 152.51°E	20	10
2	Tea Gardens, NSW	Apr. 2019	32.66°S, 152.15°E	20	–
3	Nelson Bay, NSW	Mar.–Apr. 2019, Aug. 2019	32.72°S, 152.15°E	30	10
4	Sydney, NSW	Apr. 2019	33.87°S, 151.21°E	20	–
5	Wollongong, NSW	Jan.–Mar. 2019	34.43°S, 150.8931°E	26	–
6	Kiama, NSW	Jan.–Feb. 2019	34.67°S, 150.84°E	25	–
7	Ulladulla, NSW	Jan.–Apr. 2019	35.36°S, 150.46°E	26	–
8	Eden, NSW	June–July 2019, May 2018	37.07°S, 149.90°E	20	25
9	Corner Inlet, VIC	Aug. 2019	38.78°S, 146.33°E	–	31
10	Adelaide, SA	Feb. 2020	34.93°S, 138.60°E	–	20
11	Perth, WA	Apr. 2019	31.95°S, 115.86°E	–	24

Site numbers corresponds with the numbers in Fig. 1.
NSW, New South Wales; VIC, Victoria; SA, South Australia; WA, Western Australia.

sequences from GenBank for *H. australis* (GenBank accession number KX781932.1) and *H. melanochir* (GenBank accession number HQ956051.1) by using the Basic Local Alignment Search Tool (BLAST; Altschul *et al*. 1990). Initial comparison of these two sequences showed four consistent diagnostic base differences between the species. Only sequences with a 100% match to either of these reference sequences at these four diagnostic bases were assigned to a species group. Twelve sequences appeared to have mixed identity on the basis of these four diagnostic bases, including three sequences with double peaks on their chromatogram traces only at these base pair positions, and thus could not be assigned definitively to either species.

All sequences were exported in FASTA format and collapsed into haplotypes before conversion into ARLEQUIN and NEXUS file formats by using the online tool FaBox (see http://www.birc.au.dk/software/fabox; Villesen 2007). Unique haplotypes were then imported into jModelTest (ver. 2.1.10, see http://evomics.org/learning/phylogenetics/jmodeltest/; Guindon and Gascuel 2003; Darriba *et al*. 2012), which was used with an Akaike information criteria (AIC; Bozdogan 1987) to determine the best nucleotide substitution model for the sequence data. For the mtDNA haplotypes, the Tamura–Nei (TrN; Tamura and Nei 1993) model was selected for use in subsequent genetic analysis as required.

To test for differences in genetic diversity between *H. australis* and *H. melanochir*, ARLEQUIN (ver. 3.5.2.2, see http://cmpg.unibe.ch/software/arlequin35; Excoffier and Lischer 2010) was used to calculate haplotype (h) and nucleotide (π) diversity. These diversity indices were then used in an analysis of molecular variance (AMOVA) to determine global Φ_{ST} values with non-parametric permutations (n = 99 999). Pairwise Φ_{ST} statistics were generated to test the significance of differences between locations and then adjusted to correct for false discovery rate (as per Narum 2006). Fu's F_s values (Fu 1997) were also calculated to test for deviations from neutral sequence evolution, with significance being tested using 99 999 permutations.

To visualise the relationship between haplotypes, the genetics software PopART (ver. 1.7, see https://popart.maths.otago.ac.nz/; Leigh and Bryant 2015) was used to construct a median-joining network, which was produced using algorithms and settings according to Bandelt *et al*. (1999).

The corrected average Kimura two-parameter (K2P) pairwise difference between the *COI* haplotypes of both species was calculated in ARLEQUIN and divided by the number of base pairs in the sequence to determine the mean K2P percentage difference between the *COI* sequences of both species. This difference was then used to estimate the time of divergence on the basis of a molecular-clock calibration rate for fish of 1.2% *COI* divergence per million years. This calibration rate is rooted in differences between the *COI* regions of sister taxa separated by the rise of the Isthmus of Panama *c*. 3.8 million years ago (Bermingham *et al*. 1997) and has been used to estimate the time of divergence for a variety of marine fish species (Lessios 2008; Tea *et al*. 2019; Delrieu-Trottin *et al*. 2022). To confirm that the K2P mean difference and time of divergence were supported by the model of nucleotide substitution that was chosen in jModelTest to best fit our species (i.e. Tamura–Nei), this approach was repeated using the corrected average TrN pairwise difference calculated in ARLEQUIN, which produced identical results (Supplementary Table S6).

To contextualise genetic differences within the genus, the mean K2P percentage difference and approximate time of divergence was then calculated for all other species within the *Hyporhamphus* genus with available reference sequences on GenBank and compared to our two study species. All *COI* sequences over 600 bp long for 12 *Hyporhamphus* species were downloaded from GenBank, then aligned and trimmed

in Geneious Prime (Supplementary Table S7). Aligned sequences were then exported, collapsed into haplotypes, and converted to ARLEQUIN file format by using FaBox. This sequence-editing workflow was repeated for each species to construct a pairwise table of mean K2P percentage difference and approximate time since divergence between each species. These methods were then repeated using the corrected average TrN pairwise difference as above (Table S6).

Genetic analysis – nuclear *TMO-4C4*

A 450-bp segment of the nuclear DNA *TMO-4C4* gene was amplified using primers TMO_f1_5 and TMO_r1_3 and PCR conditions outlined in Streelman and Karl (1997) prior to sequencing at the Ramaciotti Centre for Genomics (see the 'Supplemental detailed genetic methods' section in the Supplementary material and Table S5). All *TMO-4C4* sequences were aligned, trimmed and visually inspected for base ambiguities in Geneious Prime and deposited in the NCBI GenBank nucleotide database (GenBank accession numbers MZ580145–MZ580399). Any alignments with ambiguous base calls were edited against their reverse complement for base confirmation, and all alignments with <70% high-quality bases were excluded from further analysis (n = 16). After exporting sequences in FASTA format, the allelic states of these nuclear sequences were inferred using the Bayesian algorithm PHASE (ver. 2.1, see https://stephenslab.uchicago.edu/phase/download.html; Stephens *et al.* 2001; Stephens and Donnelly 2003) implemented within the software DnaSP (ver. 6.12.03, see http://www.ub.edu/dnasp/; Rozas *et al.* 2017). Three runs in PHASE (100 000 iterations) with a burn-in of 10 000 all returned consistent allelic identities, and PHASE was able to resolve most alleles with 100% certainty. Individuals with <80% certainty at single nucleotide positions were removed from further analysis (n = 34 individuals, 13% of total samples). Allelic sequences were then exported in FASTA file format, collapsed into unique alleles, and converted into ARLEQUIN file format with FaBox prior to genetic analysis.

AIC analysis of unique TMO sequences in jModelTest also resulted in the selection of the TrN model, which was used in ARLEQUIN to calculate diversity indices (h and π), Fu's F_s-values and AMOVA statistics to complement those performed for the mitochondrial *COI* gene.

Morphological analysis – body morphometrics and meristics

Following formalin preservation, 14 morphometric measurements and 6 meristic counts were recorded for each whole fish. All characters measured were taken from past comprehensive studies of other *Hyporhamphus* species (Collette 1974; Banford 2010; Table 2). Measurements were recorded to the nearest 0.1 mm with digital callipers, and vertebrae were counted from the X-rays taken of each specimen at the Australian Museum, visualised using Adobe Illustrator. X-Rays were also used to corroborate the dorsal and anal fin ray counts taken directly from specimens.

Table 2. List of all meristic and morphometric characters measured on *Hyporhamphus australis* and *H. melanochir*.

Item	Abbreviation	Description
Meristic character		
Dorsal fin rays	DOR	All elements counted
Anal fin rays	ANA	All elements counted
Pectoral fin rays	P1	All elements counted
Vertebrae	VERT	Precaudal + Caudal = total includes hypural as last vertebrae
Gill rakers on first arch	RGR1	All on right side
Gill rakers on second arch	RGR2	All on right side
Morphological character		
Standard length	SL	Tip of upper jaw to caudal base
Lower-jaw length	LJL	Tip of upper jaw to tip of lower jaw
Upper-jaw length	UJL	Tip of upper jaw to where upper jaw bends
Upper-jaw width	UJW	Measured where upper jaw bends
Head length	HDL	Tip of upper jaw to posterior end of opercular membranes
Pectoral fin length	P1L	Base of uppermost pectoral ray to tip of longest ray
Dorsal fin base length	DBASE	Origin of fin to last ray
Anal fin base length	ABASE	Base length of anal fin
Pectoral–pelvic length	P1-P2	Distance from origin of pectoral fin to origin of pelvic fin
Pelvic–caudal length	P2-C	Distance from origin of pelvic fin to caudal base
Body depth 1	BD P1	At origin of pectoral fin
Body depth 2	BD P2	At origin of pelvic fin
Diameter of soft orbit	ORB	Soft orbit diameter
Preorbital length	PREORB	Corner of mouth to anterior margin of orbit

All characters and descriptions have been taken from past studies by Collette (1974), Banford (2010) and Banford and Collette (1993).

All data were partitioned into species groups for analysis in R (ver. 4.0.4, R Foundation for Statistical Computing, Vienna, Austria, see https://www.R-project.org/), on the basis of the species diagnosis obtained from the *COI* analysis. Any samples that could not be diagnosed as either species were excluded from further morphological analysis.

Multivariate principal component analysis (PCA) was performed on morphometric and meristic data separately,

to visually assess the degree of morphological difference between each species and identify which morphological characters had the greatest variation. To reduce the effect of allometric differences among individuals and species, all morphometric measurements were log-transformed and regressed against the corresponding log-transformed standard length to obtain residual values for use in PCA. Logarithmic transformations were performed to reduce the scaling effects between standard length and residual variance. Scores for the first two principal components were visualised as bivariate plots, and factor loadings of each variable were used to assess their contribution to PC variation (see Tea *et al.* (2019) for a similar transformation and analysis).

To assess significant differences in the meristic counts of each species, histograms and summary tables of the most important variables contributing to factor loadings along the first axis were constructed. The size-adjusted mean morphometric variables for each species were compared by performing an analysis of covariance (ANCOVA) on each variable. All measurements were log-transformed to meet assumptions of normality and reduce scaling effects, and any observations whose standardised residuals were >3 were considered outliers and excluded from that analysis. Bonferroni adjustments were applied to the resulting *P*-values to adjust for multiple testing. Morphological variables identified as significantly ($P \leq 0.003$) different were then visualised as boxplots to further explore differences among species and locations.

Morphological analysis – otolith shape

All otoliths were photographed using a Leica IC80 HD camera mounted on a Leica M125 dissecting microscope. Left-side sagittal otoliths were positioned distal side up with their rostrum horizontally aligned, on a black background (Fig. S1). Any otoliths that had broken in storage were pieced together under the microscope by using tweezers. Adobe Photoshop (ver. 22.2.0, Adobe Inc., San Jose, CA, USA) was used to align and stitch together these fragments to reconstruct the shape outline. In cases where the left-side otolith was missing or broken and the right-side one was intact, the right-side otolith was photographed, and Adobe Photoshop was used to flip the image to align horizontally with the other samples (see Steer and Fowler 2015 for similar methods on *H. melanochir* otoliths). Data were partitioned into species groups prior to analysis on the basis of the results from *COI* barcoding.

To extract otolith contour outlines and transform them into independent coefficients by using discrete wavelet analysis, all photographs were processed using the R package ShapeR (ver. 0.1.5, see https://cran.r-project.org/package=shapeR; Libungan and Pálsson 2015). The mean otolith shape for each species was plotted, and coefficients that showed an interaction with fish length ($n = 6$) were removed to adjust each otolith shape with respect to allometric relationships with fish length. To analyse the variation in otolith shape between the two species, a permutational multivariate analysis of variance (PERMANOVA) was applied to the length standardised wavelet coefficients by using the R package vegan (ver. 2.5.6, J. Oksanen, F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, and H. Wagner, see https://cran.r-project.org/web/packages/vegan/). Assumptions of equal variance among groups were checked with a dispersion test by using the function '*betadisper()*'. No differences were detected between the outlines of the photoshopped and non-photoshopped files (PERMANOVA, $P > 0.1$ for all locations, Supplementary Table S8). Random forest classification analysis was then performed on these coefficients by using the R package randomForest (ver. 4.6.14, see https://cran.r-project.org/package=randomForest; Liaw and Wiener 2002) to determine the error rate of classification of individuals to their species group.

Species discrimination

To assess the potential use of morphological variables as diagnostic markers, classification trees were constructed using the R packages rpart (ver. 4.1.15, T. Therneau and B. Atkinson, see https://CRAN.R-project.org/package=rpart) and rpart.plot (ver. 3.0.9, S. Milborrow, see https://CRAN.R-project.org/package=rpart.plot). Ratios were constructed among key variables identified from PCA and ANCOVA to account for differences in standard length among samples and to attempt to increase the strength of differences in variables among the species. Trees with diagnostic error rates were then constructed separately for meristic counts and ratios of key measurements to compare the probability of correct diagnosis between both methods. To reduce the impact of differences in sample size between species on probability estimates and account for potential geographic variation in *H. melanochir* morphology, only data from NSW and Victoria were used to construct classification trees (see Table 1, Sites 1–9).

Results

Genetic analysis – mitochondrial *COI*

The mitochondrial *COI* fragment for *H. australis* amplified in this study differed from that of *H. melanochir* by 0.66%. On the basis of a 1.2% per million year rate of divergence calibrated with the *COI* gene and the rise of the Isthmus of Panama (Bermingham *et al.* 1997), our study suggests that the two species may have separated *c.* 500 000 years ago. Comparison of this pairwise genetic difference with other species in the genus indicates that, of the 12 *Hyporhamphus* species found on GenBank, *H. australis*, *H. melanochir* and the New Zealand endemic *H. ihi* are the most closely

Table 3. Estimates of the percentage pairwise difference in the mitochondrial DNA *COI* sequences of *Hyporhamphus* species.

	affinis	*australis*	*dussumieri*	*ihi*	*limbatus*	*meeki*	*melanochir*	*quoyi*	*regularis*	*sajori*	*unifasciatus*	*xanthopterus*
affinis	–											
australis	8.26 (6.9)	–										
dussumieri	9.86 (8.2)	12.38 (10.3)	–									
ihi	8.27 (6.9)	**0.52 (0.4)**	12.93 (10.8)	–								
limbatus	8.03 (6.7)	12.08 (10.1)	9.96 (8.3)	12.29 (10.2)	–							
meeki	14.67 (12.2)	17.42 (14.5)	14.80 (12.3)	17.90 (14.9)	14.39 (12)	–						
melanochir	8.31 (6.9)	**0.70 (0.6)**	12.85 (10.7)	**0.52 (0.4)**	12.33 (10.3)	17.90 (14.9)	–					
quoyi	4.18 (3.5)	9.01 (7.5)	9.27 (7.7)	9.00 (7.5)	3.52 (2.9)	14.11 (11.8)	9.23 (7.7)	–				
regularis	11.47 (9.6)	14.77 (12.3)	12.81 (10.7)	14.10 (11.7)	11.23 (9.4)	18.30 (15.3)	14.32 (11.9)	10.86 (9)	–			
sajori	16.98 (14.2)	17.38 (14.5)	13.81 (11.5)	18.10 (15.1)	15.82 (13.2)	19.83 (16.5)	18.10 (15.1)	15.63 (13)	19.64 (16.4)	–		
unifasciatus	9.43 (7.9)	12.30 (10.2)	9.86 (8.2)	12.68 (10.6)	8.88 (7.2)	2.9 (2.4)	12.59 (10.5)	8.3 (6.9)	12.45 (10.4)	16.41 (13.7)	–	
xanthopterus	6.22 (5.2)	12.90 (10.7)	14.13 (11.8)	12.68 (10.6)	14.02 (11.7)	19.82 (16.5)	12.90 (10.7)	10.59 (8.8)	16.49 (13.7)	20.99 (17.5)	14.34 (11.9)	–

All sequences used to construct the table are taken from GenBank and BOLD (see Table S7). The top value in each cell represents the Kimura two-parameter (K2P) percentage difference between the sequences (%), and the bottom values in parentheses are approximations of the time since divergence (Ma) based on an estimated *COI* mutation rate of 1.2% per million years. Names are shortened to species. Bold values indicate differences among *Hyporhamphus australis*, *Hyporhamphus melanochir* and *Hyporhamphus ihi*.

related on the basis of *COI* (Table 3). Because there was only a single haplotype present on GenBank for *H. australis* and *H. melanochir*, the difference between these samples was slightly higher than the results from our overall sampling efforts (0.7% difference, indicating separation *c*. 600 000 years ago, Table 3). Average conspecific (within-species) differentiation at *COI* was only 0.03% for *H. australis* and 0.04% for *H. melanochir*.

The low genetic divergence observed among and within species groups is supported by the median-joining haplotype network (Fig. 2*a*). Within each species group, one major haplotype was dominant (Fig. 2*a*). Haplotypes among species consistently differed at the four diagnostic nucleotides, whereas haplotypes of conspecifics differed by no more than two nucleotide substitutions. No haplotypes attributed to *H. australis* were found outside of NSW; however, the dominant *H. melanochir* haplotype (Fig. 2*a*) was found in NSW regions outside of their overlapping distribution at Eden (n = 4 samples, one each from Tea Gardens, Nelson Bay, Kiama, and Ulladulla). Aside from these four samples, Eden was the only location with a complete overlap in haplotypes from both species (Fig. 2*a*). The *COI* sequence data showed 13 haplotypes for *H. australis* and six for *H. melanochir*, with a small number of intermediate haplotypes (n = 3) that could not be attributed to either species group. One haplotype was found across multiple locations (Tea Gardens, Eden, Victoria, and Adelaide), and two were found only in Eden. Of the two intermediate haplotypes unique to Eden, one haplotype shared by three individuals had double peaks on their chromatogram traces at all diagnostic bases between the two species.

AMOVA confirmed that there were significant genetic differences between the two species that explained 95% of the variation in haplotype diversity (overall Φ_{ST} = 0.95, P < 0.001, Supplementary Table S9). Population pairwise tests among all samples grouped by location showed that Eden was the only population that differed significantly from populations at all other geographic sites (Fig. 3, Supplementary Table S10). Victorian, South Australian, and Western Australian populations were significantly different from all NSW populations, but not from each other. All NSW populations (except for Eden) were also not significantly different from one another. Indeed, within each species group, there were no significant differences among locations (*H. australis:* overall Φ_{ST} = 0.005, P = 0.153; *H. melanochir:* overall Φ_{ST} = 0.04, P = 0.057; Table S9).

When comparing across all locations, the haplotype diversity (h) of *H. melanochir* was approximately 50% higher than that of *H. australis*, whereas nucleotide diversity (π) was the same for both species (Supplementary Table S11). Tests of neutrality showed negative and significant Fu's F_s values for both species; within each species group, values of h, π and F_s varied across locations (Table S11). Within the *H. australis* group, the populations sampled at Forster, Nelson Bay, Wollongong, and Eden all had higher haplotype diversity and

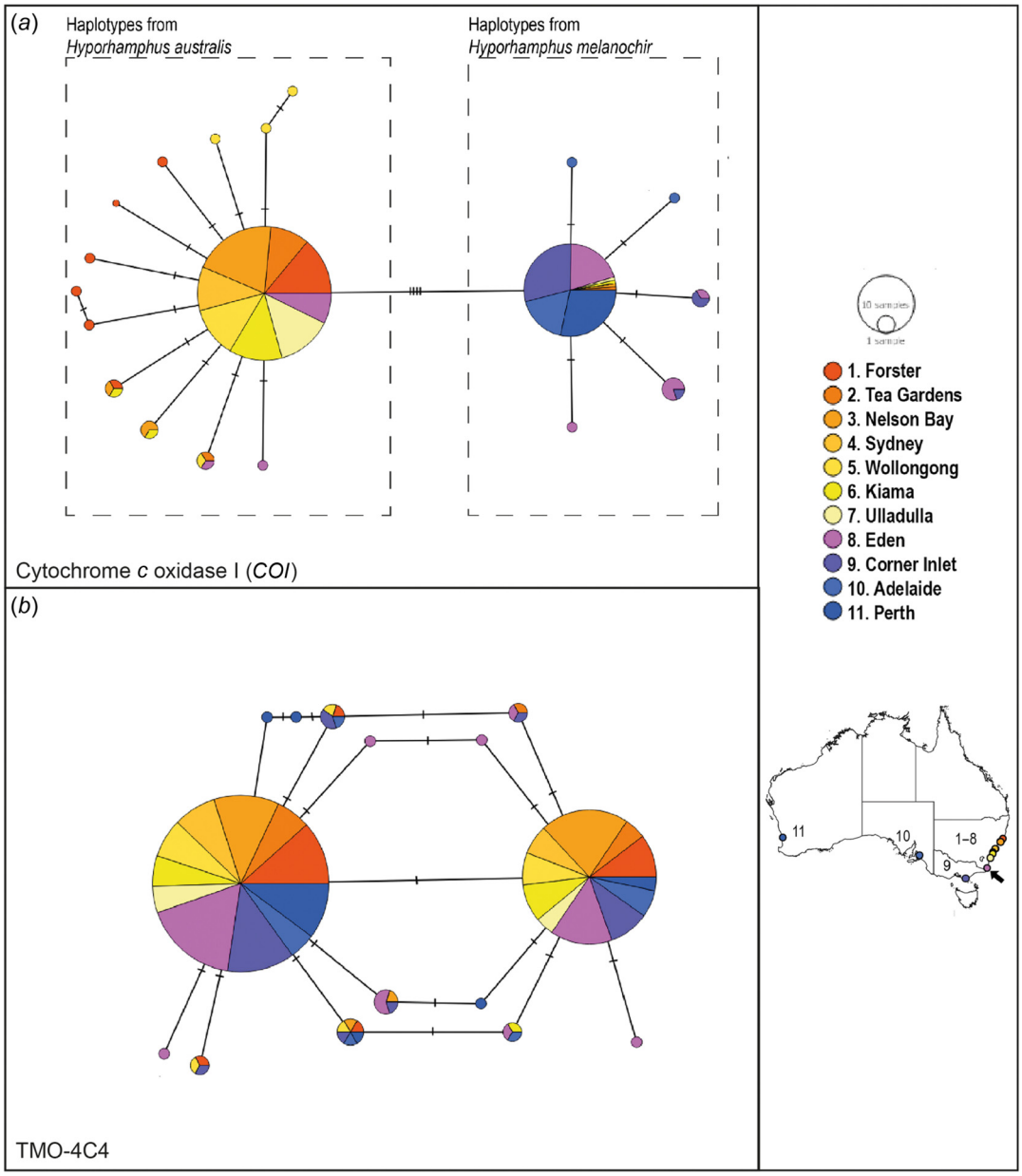

Fig. 2. Median-joining network showing relationships (*a*) among mitochondrial *COI* haplotypes and (*b*) nuclear *TMO-4C4* alleles for *Hyporhamphus australis* and *H. melanochir*. Each circle represents a haplotype or allele, and the circle size indicates the frequency of that haplotype or allele. The colours within circles indicate the location of origin of that haplotype or allele. Location numbers correspond with Table 1 and the map insert (Eden, the area where both species distributions overlap, is indicated with an arrow). Branches and black cross-bars represent a single nucleotide change.

significant F_s values (Table S11). Within the *H. melanochir* group, Eden had the highest haplotype and nucleotide diversity, but significant F_s values were seen only in the Victoria and South Australia populations (Table S11).

Genetic analysis – nuclear *TMO-4C4*

The nuclear *TMO-4C4* sequences of 221 garfish individuals showed 15 alleles, of which 8 were found in both species, with approximately equal allelic and nucleotide diversities (Table S11). *Hyporhamphus melanochir* from Eden and Western Australia were the only groups that contained unique alleles, and had significant and negative F_s values ($F_s = -3.24$ and -3.28 respectively; Table S11).

AMOVA analysis and visualisation of the median-joining network based on the *TMO-4C4* alleles indicated that there was no significant genetic difference between species ($P = 0.09$) or locations ($P = 0.47$; Supplementary Table S12,

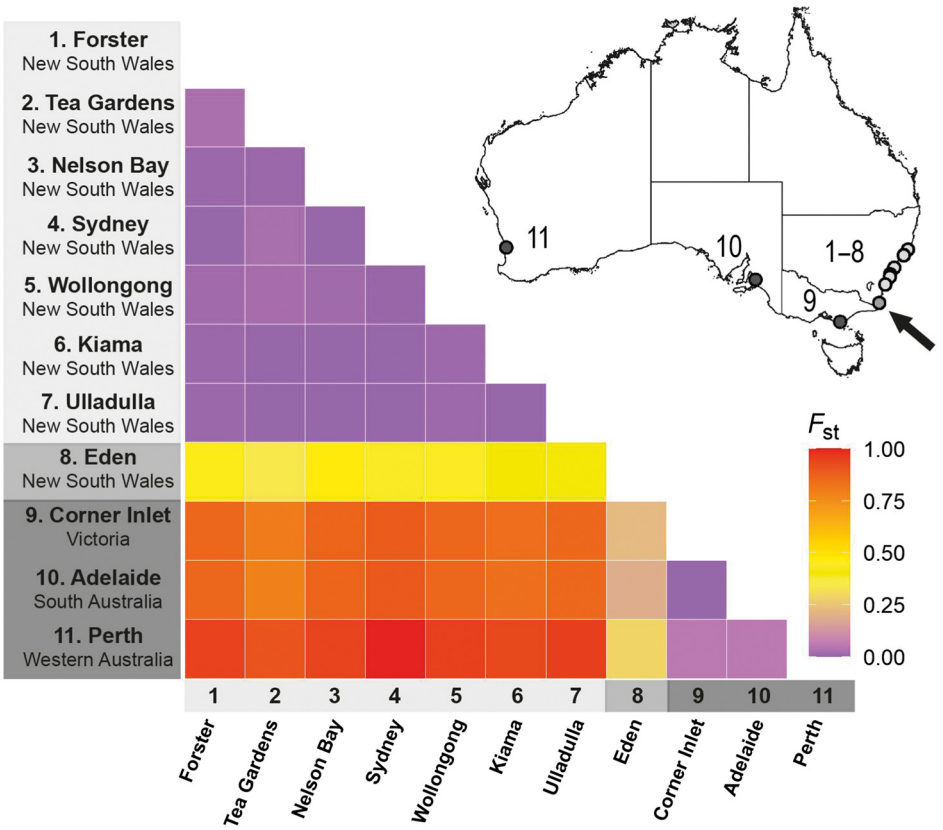

Fig. 3. Heatmap of pairwise F_{ST} values based on mitochondrial DNA *COI* sequence data for *Hyporhamphus australis* and *H. melanochir* sampled at 11 locations throughout their mainland coastal distribution in Australia. Colours correspond to F_{ST} values as indicated in the legend. Numbers along the *x*-axis correspond with sampling location numbers shown in Fig. 1 and the map insert (Eden, the area where both species distributions overlap, is indicated with an arrow). Grey boxes surrounding sampling locations indicate groups of locations that do not significantly differ from one another, but which differ significantly from other grey location groups ($P \leq 0.003$, Table S10).

Fig. 2*b*). Two major alleles dominated at all locations across both species, which differed by only one nucleotide substitution (Fig. 2*b*).

Morphological analysis – body morphometrics and meristics

PCA showed clear distinctions between the meristic characters of the two species (Fig. 4*a*). Individuals were separated into species groups along the PC 1 axis, which accounted for 41.4% of the total variance (Fig. 4*a*). Gill raker counts on the first and second arch contributed the most to the factor loadings of PC 1 (−0.8 and −0.87 respectively; Supplementary Table S13).

The PCA of morphometric characters indicated separation between the species but with a considerable amount of overlap along the PC 2 axis, which accounted for 16.4% of the total variance (Fig. 4*b*). Preorbital length and upper-jaw length both contributed the most to factor loadings of PC 2 (−0.74 and −0.65 respectively; Supplementary Tables S14, S15).

Mean gill raker counts differed between the two species, with *H. australis* presenting higher mean gill raker counts on both the first and second gill arches (Fig. 5, Table 4). Mean vertebral counts also differed between the species; however, *H. melanochir* showed a significant amount of within-species variation among locations (Fig. 5). Vertebral counts were lower across the entire range of *H. australis v.* those for *H. melanochir* individuals from the geographically closest locations (Eden and Victoria), but there was no clear difference between vertebral counts of *H. australis* and *H. melanochir* from more distant locations (South Australia and Western Australia; Fig. 5, Table 4). Although dorsal fin rays were identified in PCA as contributing to factor loadings along the PC 1 axis, there was a high degree of overlap between the two species (Fig. 5).

ANCOVA showed that, of the 13 morphometric measurements examined, there were significant differences between

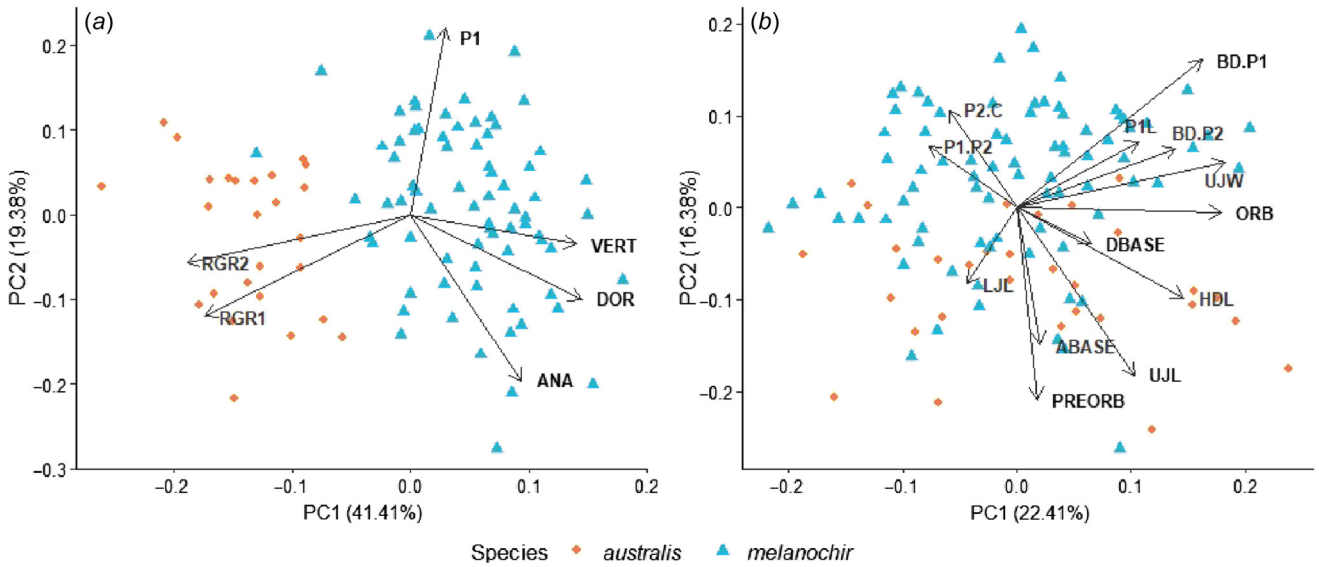

Fig. 4. Plots of the first two principal components from a principal component analysis (PCA) of (*a*) six meristic characters and (*b*) 13 morphometric characters based on 30 specimens of *Hyporhamphus australis* and 81 specimens of *H. melanochir*. Arrows and labels indicate variables, names correspond with characters in Table 2. Arrow length is proportional to the contribution of variables to PC variation.

the group means of only three measurements, namely, head length, upper-jaw length, and preorbital length ($P < 0.003$; Supplementary Tables S16, S17). *Hyporhamphus australis* was found to have a larger head size than was *H. melanochir*, which was driven by its longer upper-jaw and preorbital length (Fig. 6). There was no significant difference in the orbital diameter or distance between the orbit and the posterior end of the opercular membrane between the species ($P = 0.5$ and 0.08 respectively; Tables S16, S17). ANCOVA also showed a significant difference in the mean pectoral fin length between the species ($P < 0.003$; Tables S16, S17), but owing to the small effect size and high degree of overlap in the range of lengths for each group, this trait was deemed to be an unsuitable diagnostic feature (ges = 0.08, Tables S16, S17, Fig. S2).

In general, both species maintained these differences in characteristics even within areas where their distributions overlapped (Eden, Fig. 6). Difference in upper-jaw length was more pronounced between species in Eden than between species from more geographically distant populations, but there was more overlap in preorbital length between species in Eden than between species from other locations (Fig. 6). Although the mean upper-jaw and preorbital lengths were significantly different between the two species, there was still a considerable amount of overlap in these characters (Fig. 6).

Morphological analysis – otolith shape

PERMANOVA showed significant variation in otolith shape between the two species ($P = 0.001$; Supplementary Table S18). Wavelet reconstruction of mean otolith shape showed that the otoliths of *H. australis* tend to have a slightly longer, more pointed rostrum than do those of *H. melanochir* (Fig. 7).

Random forest classification based on wavelet coefficients resulted in assignment to species with a high degree of success (~88%; Supplementary Table S19). The within-class error was substantially lower for *H. australis* than *H. melanochir* (5.49 and 28.99% respectively; Table S19), suggesting a higher variability in *H. melanochir* otolith shape.

Species discrimination

Classification trees using data from NSW and VIC populations showed that, although it is possible to distinguish between the species on the basis of ratios of head characteristics, gill raker counts remain the most accurate method of diagnosis between the two species (Fig. 8). The number of gill rakers on the second arch alone were enough to distinguish between the species with ~92% accuracy, whereas ratios of four separate measurements had a lower accuracy rate of ~89% (Fig. 8). These classification trees provide a more accurate species diagnosis than does otolith shape (Table S19); however, owing to the higher degree of overlap in morphological measurements between the species (see Fig. 6), gill rakers should be used to distinguish between species wherever possible.

Discussion

This study has demonstrated the use of an integrative taxonomic approach to separate two cryptic halfbeak species

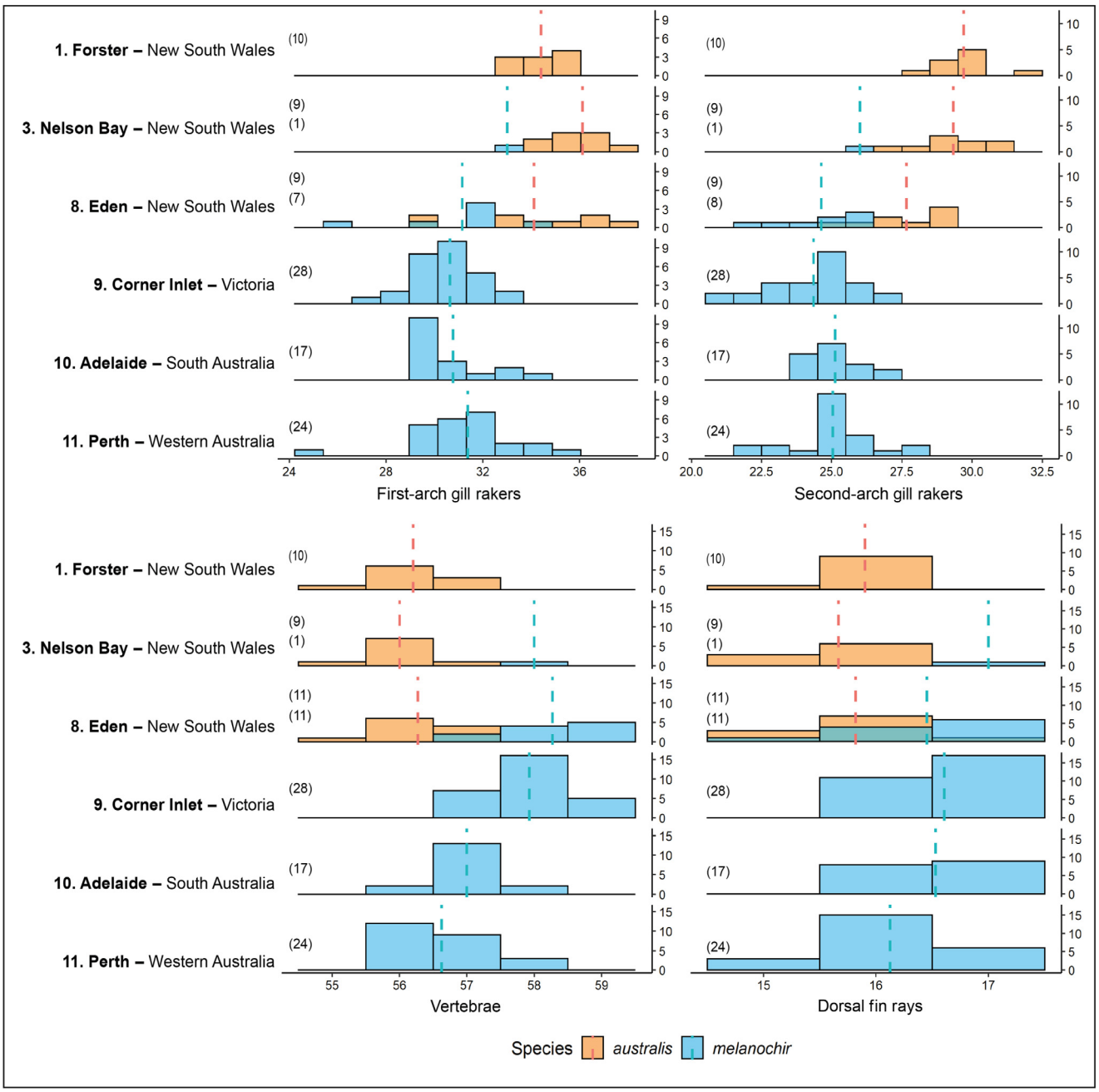

Fig. 5. Histograms showing the difference in gill rakers, vertebrae, and dorsal fin rays of *Hyporhamphus australis* and *H. melanochir* samples across locations. Numbers next to each location name correspond to site numbers in Fig. 1 and Table 1. Dashed lines indicate group means. Numbers in parentheses indicate sample sizes.

Table 4. Summary of differences in meristic characters between *Hyporhamphus australis* and *H. melanochi.*

Species	Gill rakers (first arch)	Gill rakers (second arch)	Vertebrae	Dorsal fin rays
Hyporhamphus australis	Usually ≥34 (75% of 28 specimens examined)	Usually ≥27 (89% of 27 specimens examined)	55–57	Usually 15–16
Hyporhamphus melanochir	Usually ≤33 (93% of 78 specimens examined)	Usually ≤26 or less (91% of 78 specimens examined)	57–59 (Eden and Victoria) 56–58 (Adelaide and Perth)	Usually 16–17

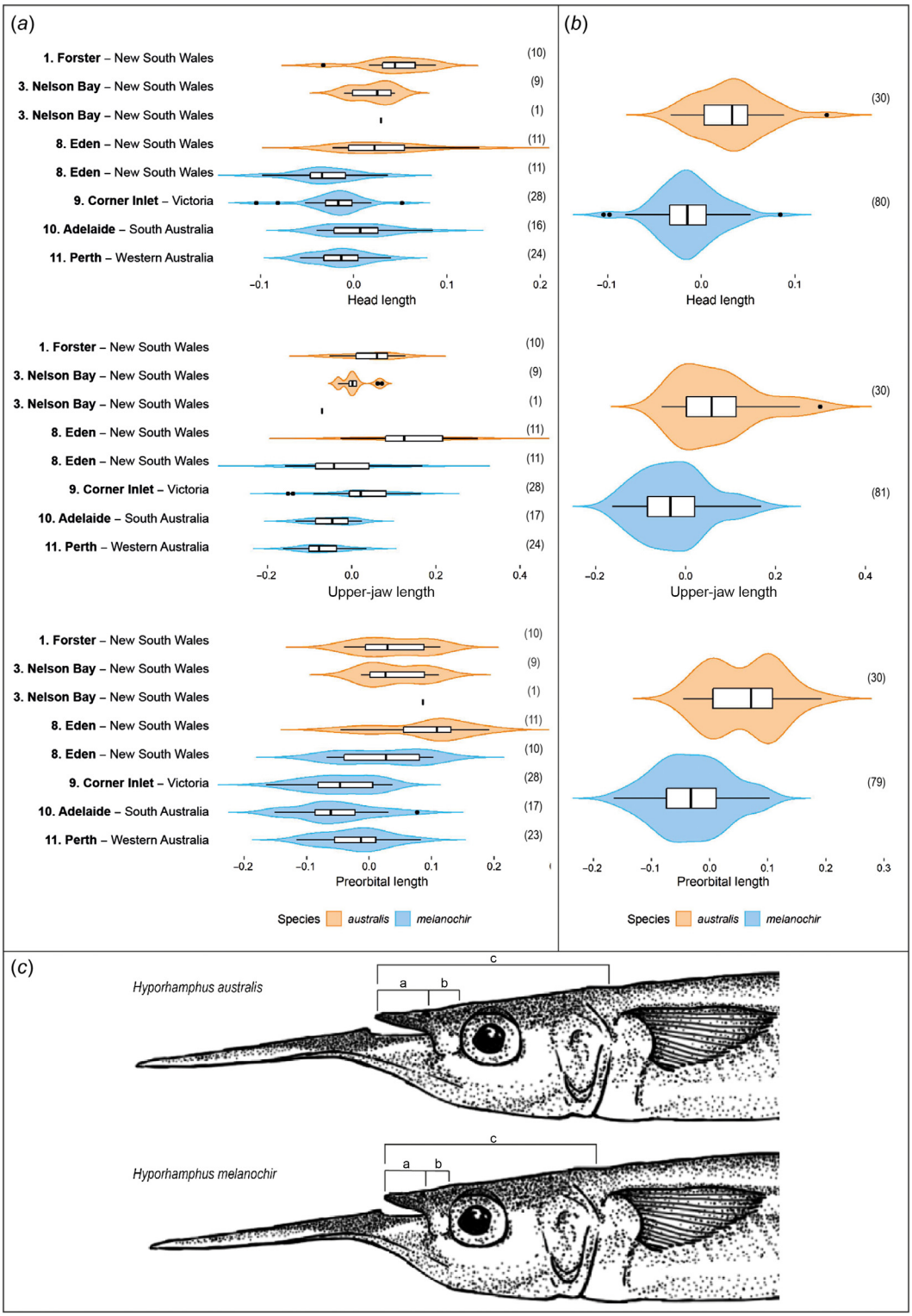

Fig. 6. Violin plots displaying the differences between the head length, preorbital length and upper-jaw length of *Hyporhamphus australis* and *H. melanochir* grouped by (*a*) location and (*b*) pooled in species groups, standardised to account for differences in standard length. Plots display the residuals of log-measurements plotted on log-length, not raw measurements. Shaded areas represent kernel-utilisation distributions. Numbers next to each location name correspond to site numbers in Fig. 1 and Table 1. Numbers in parentheses indicate sample size. Measurements align with (*c*) the diagram of the heads of a typical *H. australis* and *H. melanochir* specimen displaying differences in (*a*) the upper-jaw length, (*b*) preorbital length, and (c) head length. Drawn in Adobe Photoshop by I. Riley.

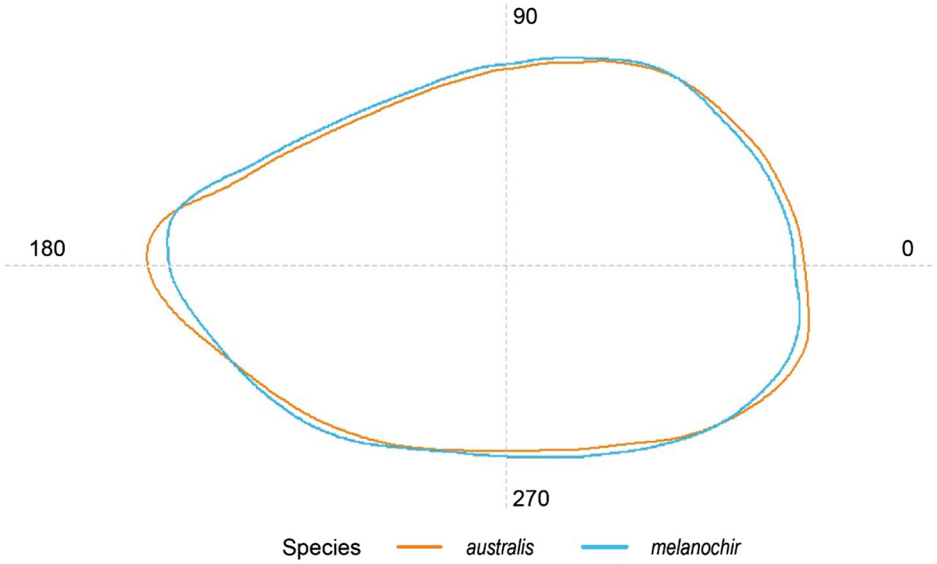

Fig. 7. Mean otolith shape based on wavelet reconstruction for *Hyporhamphus australis* ($n = 176$) and *H. melanochir* ($n = 86$). Numbers represent angles in degrees based on polar coordinates. The centroid of the otolith (centre of the cross) is the centre point of the polar coordinates.

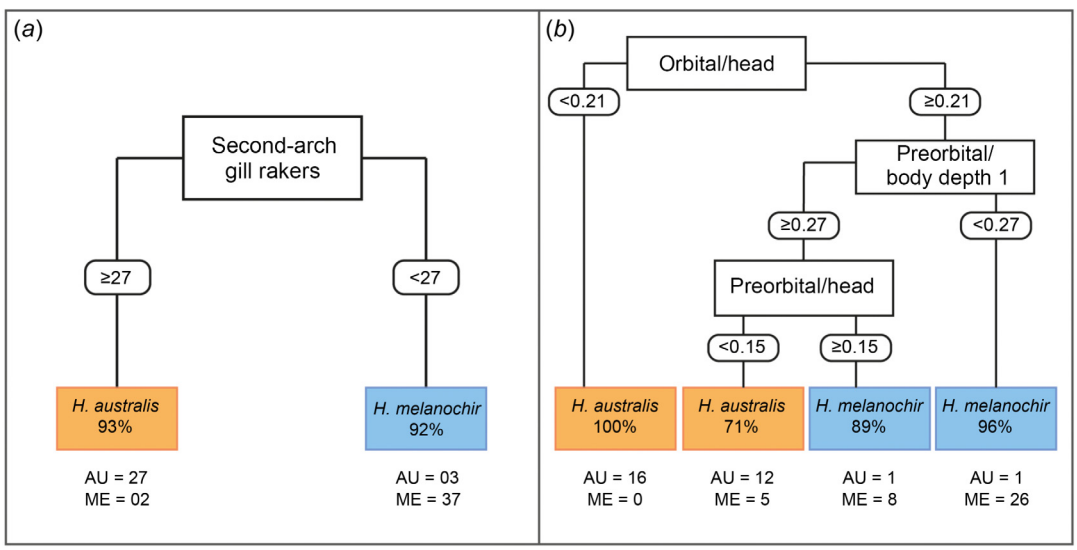

Fig. 8. Classification trees for diagnosis between *Hyporhamphus australis* and *H. melanochir* using (*a*) second-arch gill raker counts and (*b*) ratios of body measurements. Each tree should be read from top to bottom and shows the number or value of each trait that is usually found in individuals from each species (e.g. Fig. 8*a* shows that *H. australis* usually has 27 or more gill rakers on their second gill arch, whereas *H. melanochir* usually has fewer than 27). The numbers on each branch correspond with the count or measurement (mm) of the label (trait) above it. The base nodes indicate the probability that samples belong to the fitted species class. The numbers below each node indicate the number of *H. australis* (AU) and *H. melanochir* (ME) samples assigned to each predicted species class.

with overlapping distributions. *Hyporhamphus australis* and *H. melanochir* are a cryptic halfbeak species pair with consistent differences in their mtDNA *COI* gene regions that align with differences in the number of gill rakers, head length, and otolith shape. Although these differences are modest, they reinforce previous findings (Collette 1974) and confirm the taxonomic status of both species. Our study has provided new methods to discriminate between the species and has confirmed that their distributions overlap in southern NSW. The identification of both *H. melanochir* and *H. australis* in landings from southern NSW has important implications for fisheries management, both providing new morphological methods to determine the species caught in landings around Eden and highlighting

the potential need for changes into catch regulation to accommodate the growth rates and life-history strategies of *H. melanochir* stocks in NSW.

Genetic differences

The shallow divergence in the mtDNA *COI* gene region and the lack of difference in the nuclear *TMO-4C4* gene suggests that these two species have only recently diverged from each other relative to other species in the *Hyporhamphus* genus. A relatively short period of evolutionary isolation may not have allowed enough time for differences to develop in the nuclear genome since mtDNA has an effective population size approximately one-quarter that of nuclear markers (Hurst and Jiggins 2005) and, therefore, mutates at a faster rate. The low levels of genetic divergence identified among *H. australis*, *H. melanochir* and the New Zealand endemic species *H. ihi* suggest that these three species have only recently diverged from one another on an evolutionary time scale.

The estimated time point of divergence *c*. 500 000 years ago is consistent with the assumed allopatric speciation of *H. australis* and *H. melanochir* across the Bass Strait (Collette 1974). During periods of low sea level associated with Plio-Pleistocene glacial maxima, the Bassian Isthmus is thought to have acted as a land bridge connecting Tasmania to south-eastern Victoria (Dartnall 1974; Lambeck and Chappell 2001). The hypothesis that the Bassian Isthmus created a biogeographical barrier fragmenting marine populations (Hedley 1904) is supported by genetic signatures of historical isolation in other marine species (DiBattista *et al.* 2014; Wilson *et al.* 2017). Since complete submergence of the Isthmus *c*. 14 000 years ago (Lambeck and Chappell 2001), a combination of demersal spawning and contemporary oceanographic features may have sustained these patterns of historical vicariance. The complex convergence of the East Australian Current (EAC) and South Australian Boundary Current has been shown to limit colonisation and dispersal in other marine species separated historically by the Bassian Isthmus (Waters 2008; Colton and Swearer 2012). Both *H. melanochir* and *H. australis* are demersal spawners, and their substrate-adhesive eggs and well developed larvae may increase the likelihood of early life-stage retention within seagrass habitats (Jones *et al.* 2002; Stewart *et al.* 2005), thus likely limiting passive dispersal by coastal currents.

Both *H. australis* and *H. melanochir* are found concurrently in southern NSW (Eden), making this an area of secondary contact between these cryptic species. The formation of hybrid zones resulting from secondary contact is not as rare as previously considered, especially in closely related marine species at the borders of biogeographic provinces (Hobbs *et al.* 2009, 2022; Montanari *et al.* 2014). Eden is located on the extremities of two such biogeographic provinces, namely, the eastern Peronian and southern Maugean, in an area of transition between warm and cool temperate waters (Poore and O'Hara 2007). Although little research has been conducted into the hybridisation of other marine fish around Eden, genetic signatures of introgression resulting from secondary contact after isolation have been discovered within the cuttlefish species *Sepia apama* in southern NSW (Kassahn *et al.* 2003). Eden is also an area of overlap of two distinct stocks of the commercially important snapper *Chrysophrys auratus*, with the suggestion of admixture and interbreeding (Morgan *et al.* 2018) around this area. Hybridisation at Eden was first suggested by Collette (1974) and is supported by the presence of individuals in this study with intermediate mtDNA haplotypes in Eden and adjacent areas of NSW. Four samples identified as *H. melanochir* from NSW may also represent backcrossed individuals, but this cannot be confirmed because of the lack of diagnostic nuclear DNA differences between the species. Future studies should focus on the development of a panel of single nucleotide polymorphism (SNPs) markers, because their high abundance and genomic coverage often enable the detection of fine-scale genetic differences, hybridisation, and introgression between overlapping species (Gaither *et al.* 2015).

Species discrimination

The largely allopatric distributions of *H. australis* and *H. melanochir* means that species identity can almost always be determined from an individual's location of origin. However, the confirmation of a spatial and temporal species distribution overlap from the 56 km of coastline surrounding Eden, as well as the presence of a *H. melanochir* specimen from ~500 km north of their previous known range, makes diagnostic criteria essential for species identification.

Counting gill rakers remains the most reliable morphological method to distinguish these two species, and gill raker numbers should be retained as their standard diagnostic feature. These small structures are difficult to count without training and the aid of a microscope, but provide higher accuracy than morphological ratios. We recommend that gill raker counts should be integrated into the current landings monitoring conducted by NSW DPI, to better understand the proportions of both species from catches in southern NSW. Morphological ratios can also be used to discriminate between specimens in circumstances (e.g. in the field) where gill rakers may be difficult to accurately count. However, these ratios should be used with caution because of the unknown level of phenotypic plasticity in the species' morphological traits and their larger degree of overlap. Owing to the low number of individuals in this study from Eden ($n = 25$), the decision trees contain data from samples across NSW and Victoria; therefore, future studies should aim to use a much larger sample size taken across all seasons in the area of overlapping distributions.

Morphological difference

The slight morphological differences found between the species and the lack of integration of characters from the closest geographic populations support a hypothesis of recent divergence and reinforce previously published findings (Collette 1974). Most meristic and morphological traits in fish are heritable, but they are also influenced by environmental factors affecting diet, ontogeny, and growth (Swain *et al.* 2005). Differences in gill raker number, jaw length, and skull structure in fish species are often attributed to adaptive divergence and radiation, because these functional morphological traits are directly related to feeding behaviour and trophic niche use (Harrod *et al.* 2010; Parsons *et al.* 2011). Both *H. australis* and *H. melanochir* are omnivorous, feeding on *Zostera* seagrass and hyperbenthic crustaceans (Table S2), and differences in prey size and availability across their distributions may be a factor driving the differences in their gill raker counts, and upper-jaw, and preorbital lengths.

Vertebrae numbers are highly heritable in fish but are also influenced by environmental conditions during ontogeny (Swain *et al.* 2005). The high variability in vertebral counts within *H. melanochir* may be a plastic response to abiotic differences across the species latitudinal distribution, because differences found in vertebral counts are consistent with an increase in the number of fish vertebrae with an increasing latitude (i.e. Jordan 1891). The mechanism behind this pattern is unknown, but it is likely to be due to a combination of a longer ontogenetic development and larger body sizes in cooler waters (reviewed by McDowall 2008).

Our study also found significant differences in the mean otolith shapes between the two species. As is the case for other morphological features, both environmental and genetic influences have been shown to induce changes in otolith shape (Vignon and Morat 2010). This may be due to the direct and indirect effects of these factors on growth rates, which have been shown to significantly affect otolith shape in other fish species (Stransky 2014; Rodgveller *et al.* 2017). Differences in growth rates between the species may therefore contribute to their different otolith shapes, with *H. australis* having a faster growth rate than has *H. melanochir* (230 mm FL and 160–180 mm FL at 1 year of age respectively; Jones *et al.* 2002; Stewart and Hughes 2007; Table S2).

Taxonomic considerations

The corroborative genetic and morphological differences between *H. australis* and *H. melanochir* are reinforced by differences in their growth and life history. *Hyporhamphus australis* achieves smaller maximum sizes overall but grows faster and reaches maturity at smaller sizes and younger ages than does *H. melanochir* (Jones *et al.* 2002; Stewart *et al.* 2004; Table S2). Both species also have seasonal differences in their reproductive peaks, although there is a small degree of overlap owing to their protracted spawning seasons (Jones *et al.* 2002; Stewart and Hughes 2007; Table S2). Together, these differences clearly show that *H. australis* and *H. melanochir* are two distinct groups of fish for management purposes; however, the question remains as to whether these fish should be considered different species.

There are three reasons that could potentially be used to justify the revised classification of these populations as subspecies or stocks rather than species. First, the degree of genetic differentiation between this species pair (average K2P percent difference = 0.66%) is much lower than that of other species in the *Hyporhamphus* genus (Table 2) and among other congeneric species found in Australia (average K2P percentage difference = 9.93%; Ward *et al.* 2005). Second, the presence of intermediate *COI* haplotypes suggests hybridisation and potential introgression between the species, which goes against the criteria of reproductive isolation, which is needed to delimit species boundaries under the biological species concept (Mayr 1942). Finally, the morphological features that do distinguish the species have very small degrees of difference and exhibit some overlap.

However, a subspecies classification of *H. australis* and *H. melanochir* does not consider the multiple lines of evidence found in our study that separate them, and this reclassification would go against standards employed across fish taxonomy and within the *Hyporhamphus* genus. Although the average K2P percentage difference between *H. australis* and *H. melanochir* is relatively low, their between-species difference (0.66%) is ~18× higher than their average intraspecific differences (0.03 and 0.04%, respectively). Comparing these interspecific and intraspecific differences can provide a threshold to delimit potential species boundaries. Hebert *et al.* (2004) proposed a standard threshold of interspecific difference at 10× that of intraspecific difference to flag provisional species, which would suggest *H. australis* and *H. melanochir* are different species, but a threshold of intraspecific to interspecific difference alone is not enough evidence to justify their species status (Moritz and Cicero 2004). The taxonomic status of this cryptic species pair is instead confirmed by the corroboration between their genetic separation, differences in morphology and life history, as well as the maintenance of distinct traits even in geographically proximate locations. Subspecies classification would be an unsuitable assignment in this case because it is rarely used in fish taxonomy and is usually based only on discrete (i.e. allopatric) distributions (Collette 1974; Haig *et al.* 2006).

Implications

The distributional overlap of *H. australis* and *H. melanochir* in Eden has significant implications for local fisheries

management. Although the increase in lampara net mesh size from 25 to 28 mm and a reduction in fishing effort in NSW between 2004 and 2015 has successfully reduced the fishing mortality of *H. australis* (Broadhurst *et al.* 2018), these practices are not designed to accommodate the different age and size at maturity of *H. melanochir* (Table S2). The study that determined the optimal mesh size for *H. australis* also found that a larger 32-mm mesh size selected 50% of fish at 24.5 cm FL, which would effectively prevent the retention of juveniles from both species; however, this mesh type also resulted in lowered catch rates and damage to export quality fish (Stewart *et al.* 2004). Going forward, fisheries management in Eden will need to weigh the potential benefits of further increasing the minimum mesh-size limits to protect immature *H. melanochir* against the negative effect this could have on fishery catch rates.

Our study was able to show the presence of *H. melanochir* in Eden only in winter, and further research is needed to establish whether this presence is maintained year-round and explore the potential for spawning overlap. Future studies should also aim to examine traits from freshly caught fish, because the lower jaws of most of our study specimens snapped while frozen and these measurements were unable to be included in analysis. Collette (1974) noted that *H. australis* has a relatively longer lower jaw than does *H. melanochir* at larger sizes, but did not find a significant difference in their mean lengths.

The impact of these management implications may seem localised, but they could become much broader in the face of potential climate change-driven shifts in species distributions. The warming rates of western boundary currents such as the EAC are two to three times faster than that of the global sea-surface mean, and current flows are also intensifying (Wu *et al.* 2012; Malan *et al.* 2021). Changing EAC flows could shift the range of *H. australis*, which already moves up and down the NSW coast following seasonal fluctuations in the strength and temperature of the EAC (Stewart and Hughes 2007), which pushes the species further south across management jurisdictions. Similar range shifts have already been observed for many other species in this region (reviewed by Gervais *et al.* 2021). Although the smaller size and age at maturity of *H. australis* would likely mean that Tasmania's current management strategies for *H. melanochir* are conservative enough to protect the juveniles of both species, their introduction may have unforeseen impacts on the genetic or population structuring of local stocks. Even if separate management strategies are difficult or ineffective to implement within a mixed fishery, exploring the potential presence of both species in both Eden and Tasmania can give a better assessment of the true sustainability of the fishery to all stocks present, as is currently recommended for other fisheries that contain cryptic species complexes (Huveneers 2006; Saha *et al.* 2017).

Our study demonstrated the need for discrimination of cryptic species at multiple levels to validate species-level taxonomic assignment. When comparing these two species at a morphological and functional level, there are consistent differences between them that validate a distinct taxonomic classification. As De Queiroz (2007) showed in advocating for a unified species concept, these differences are properties that divergent species acquire in the process of lineage separation, which together provide evidence of speciation regardless of traditional species concepts. Studies into cryptic species should adopt similar integrative methods to those employed here, to reinforce species boundaries and aid in conservation and management.

Supplementary material

Supplementary material is available online.

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Data availability. The genetic sequence data underlying this article are available in the GenBank Nucleotide Database at https://www.ncbi.nlm.nih.gov/genbank/ (accession numbers MZ575768–MZ576070 and MZ580145–MZ580399). All other data are available from GitHub at https://github.com/Indy-Riley/Hyporhamphus_Data_Repository.

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