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A Comparison of Roe Development and Somatic Growth for Different Cryptic Species of *Mugil Cephalus* Cultured in Tropical Taiwan

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ABSTRACT

The flathead mullet, *Mugil cephalus,* is a commercially important fish for both fisheries and aquaculture in Taiwan. Based on genetic analysis, three cryptic species of *M. cephalus* were identified inhabiting the Northwest Pacific (namely NWP1-3). These species present a sympatric distribution in Taiwanese waters and studies showed that each cryptic species has its own unique reproductive characteristics. The roe development potential of the species, therefore, has become a critical selection criterion for local aquaculture. In this study, the roe development and somatic growth of different *M. cephalus* cryptic species were investigated from a local aquaculture pond in tropical Southern Taiwan with fingerlings from nearby estuarine waters. All the sampled fish were identified as NWP2 or NWP3 with the use of mitochondrial haplotype specific PCR (MHS-PCR) screening. NWP2 was the main species cultured from our study pond, which had a high gonadosomatic index (GSI 10-20%, n=15) after culturing for two years and harvesting in November. Most NWP3 sampled from the same aquaculture pond had the same age as NWP2 but with limited roe development (GSI <0.1-3%, n=11), except for the biggest NWP3, which had a similar GSI value as NWP2 and an otolith ageing of 3+ years. Both species can grow to around 408.5-483.9 mm in fork length with 930-1,630 g in weight after being cultured for two years. In order to offer a comprehensive understanding of how cryptic species differ in their reproductive biology and growth, these parameters were compared among cultured fish by other studies as well as collected data from the fields during 2005-2015. We concluded that even after 10 years, the spatial genetic structures haven't changed for *M. cephalus* cryptic species. The varied gonadal development levels of the same *M. cephalus* cryptic species cultured in different climate zones suggested their gene expression plasticity for adaptation.

Keywords: *Mugil cephalus,* cryptic species, MHS-PCR, gonadosomatic index.

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1 INTRODUCTION

The flathead mullet, *Mugil cephalus*, is a commercially important fish for both fisheries and aquaculture in Taiwan (Tang, 1964; Tung, 1981; Huang & Su, 1989). *Mugil cephalus* is also called "grey gold" because of its valuable dry roe products in Taiwan. It is also popular in Japan, countries with a coastline on the Mediterranean Sea, and South America. Based on genetic analysis, around 14 cryptic species of *M. cephalus* have been found worldwide (Durand et al., 2012; Whitfield et al., 2012; Durand & Borsa 2015); among them, three were identified inhabiting the Northwest Pacific (NWP1, NWP2, and NWP3) (Shen et al., 2011; 2022). These species present a sympatric distribution in Taiwanese waters, but were suggested to have species-specific reproductive behaviours (Shen et al., 2015). In Taiwan, NWP1 is mainly captured during its migration to the spawning grounds in the Taiwan Strait and Northeastern Taiwan, where the cold China Coast Current (CCC) meets the warm branch and main of the Kuroshio Current, respectively. The peak of the fishing season for NWP1 occurs 10 days before and after the winter solstice (Liu, 1986; Shyu & Lee, 1986; Hsu et al., 2007). The NWP2 and NWP3 were assumed to spawn near and in the estuary, respectively, according to the size of the newly recruited juveniles and the development of ovaries in the feeding grounds (Shen et al., 2015).

The aquaculture of *M. cephalus* is an important industry in Taiwan with aquaculture ponds extending from Northeastern to Southwestern Taiwan (Chien et al., 2022). The fingerlings used for aquaculture are caught from local areas or bought from fish fry dealers. Therefore, the species composition and the origin of the fingerlings will decide the species reared for the next 2-4 years since the occurrence timing and locations are different among cryptic species (Shen et al., 2015). The roe development performance and their adaptation ability in subtropical and tropical aquaculture ponds are important considerations for local aquaculture. For instance, the elevation of water temperature was found to affect the gonadal development of the cold-water minnow *Rhynchocypris kumgangensis* (Im et al., 2016).

Fish fecundity generally increases with female age simply as a function of body size because a larger body cavity allows for the development of larger ovaries. Previous studies indicated that the NWP2 females in subtropical aquaculture ponds in Taiwan have smaller body sizes but with larger developed ovaries regardless of the cultivation time, whereas the NWP1 females have larger body sizes with smaller ovaries until the 3rd year (Chien et al., 2016; 2017). As for NWP3, the females were found to have significantly larger body sizes and ovary weights than the NWP2 females under the same aquaculture conditions after culturing for three years (Chien et al., 2018). This study aims to compare the roe development of different *M. cephalus* cryptic species cultured in tropical Taiwan and to test two hypotheses: (1) Do spatial and temporal variations affect the genetic structure of *M. cephalus* cryptic species and are they still detectable in aquaculture ponds? (2) Do the variations of aquaculture conditions affect the plasticity of gene expression between two cryptic species of flathead mullet?

2 RESEARCH METHODS

2.1 Sampling design and biological measurements

In order to compare the roe (ovary) development and somatic growth for different *M. cephalus* cryptic species cultured in tropical Taiwan, mullets reared for two years (Nov. 2017-Nov. 2019) were sampled from an aquaculture pond in Checheng, Pingtung County (Figure 1), which is the most southern aquaculture area for such fish in Taiwan. Fork length (FL), total length (TL), body weight (BW), and gonad weight (GW) of the 27 cultured sub-adult to adult fish were measured. For each individual, pectoral fin clips were collected and preserved in 95% ethanol for cryptic species identification; in addition, otoliths were removed for age confirmation.

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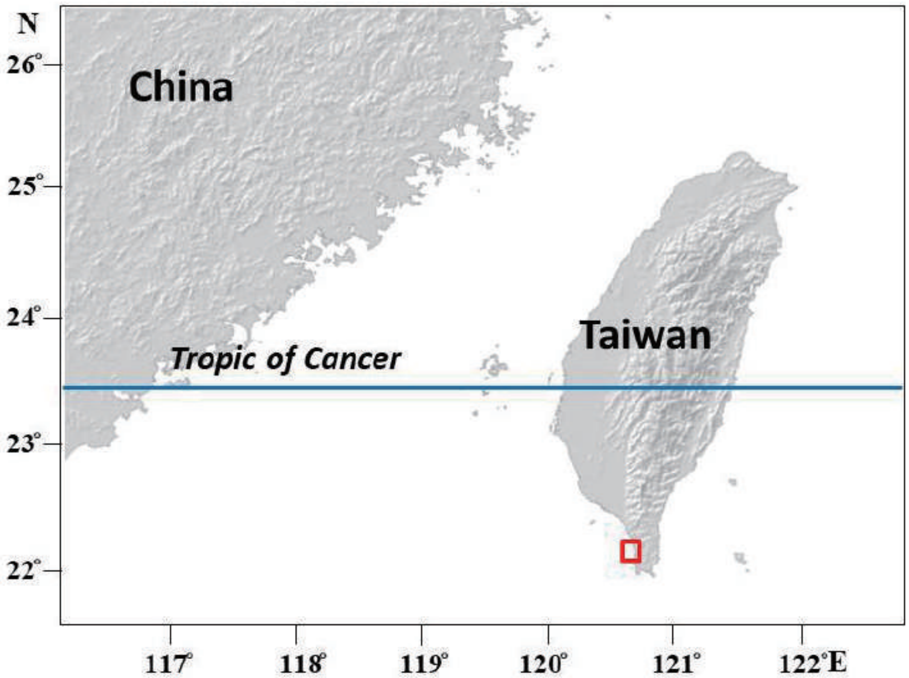

Figure 1. Sampling location of a tropical *M. cephalus* aquaculture pond (Red rectangle) in Checheng, Pingtung.

2.2 DNA extraction and polymerase chain reaction

The mitochondrial cytochrome c oxidase subunit I (COI) gene was commonly used for DNA barcoding to identify organisms based on an analysis of the variations within sequence (Hebert et al., 2003). A multiplex COI haplotype-specific PCR (MHS-PCR) method for identifying the cryptic species of *M. cephalus* was developed in a previous study (Shen et al., 2011). Genomic DNA (gDNA) was extracted from the fin clip tissue using an EasyPure Genomic DNA Kit (Bioman), preserved in ddH$_2$O, quantified and diluted to 1 ng/μL for further polymerase chain reaction (PCR) use.

PCR was conducted using an AZTECH Thermal Cycler with a 20 μL reaction volume containing 0.2 μm of each dNTP (Invitrogen 10mM mix), 2 μL 10× PCR buffer (Bioman), 0.5 μM forward and reverse primers (10mM), 0.2 U Taq DNA polymerase (Bioman), and 2.0 μL template DNA. The PCR program was as follow: 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s after heating at 95°C for 5 min. The gel electrophoresis for PCR products was run in a 2.0% agarose gel (Bioman) and stained with BioGreen Safe DNA Gel buffer (Bioman) for band characterisation via ultraviolet transillumination. The NWP1 had a PCR product of 362 bp, and 283 bp and 549 bp for NWP2 and NWP3, respectively.

2.3 Otolith ageing

The age of flathead mullets was examined following the method described by Tzeng (2010) using sagittal otolith. The left otoliths of flathead mullets were sectioned in the ventral-dorsal direction (transversely sectioned). Subsequently, the sections were ground to the core and imaged using an optical microscope (DM-2500, Leica) equipped with a digital camera (DFC 450, Leica). The opaque zone was then read from the core to the edge along the acoustic sulcus in the transverse section of the otolith, and the annual increments were counted.

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2.4 Statistics

The differences in mean values were tested by either *t*-test or the analysis of variance (ANOVA). The relative growth of fish size was fitted by the power function $BW=aFL^b$ and differences were tested by likelihood ratio test. Gonadosomatic index (GSI) was calculated by 100GW/(BW-GW).

3 RESULTS

3.1 *Mugil cephalus* cryptic species in an aquaculture pond in tropical Taiwan

The rapid screening method provided fast and reliable results to determine the cryptic species of *M. cephalus* and their percentage composition in an aquaculture pond in tropical Taiwan. All the fishes sampled from the aquaculture pond were identified as NWP2 (n=15) and NWP3 (n=12) after mitochondrial haplotype specific PCR (MHS-PCR) screening (Figure 2). After MHS-PCR screening, no individual with a 362 PCR product was detected, suggesting that no NWP1 was cultured in this tropical aquaculture pond.

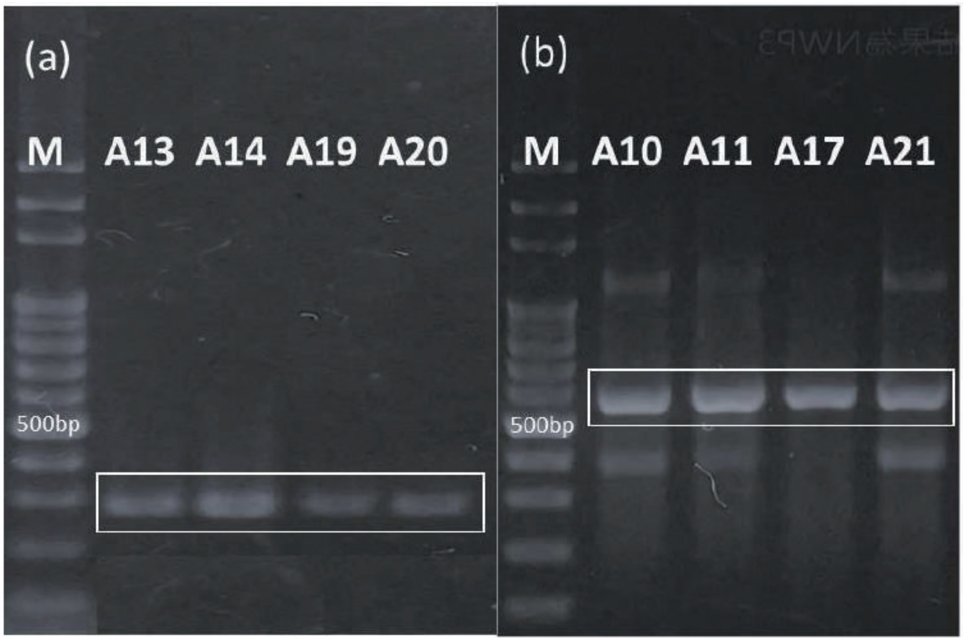

Figure 2. Examples of MHS-PCR screening for cultured *M. cephalus* cryptic species. (a) NWP2: No. A13, A14, A19, and A20; (b) NWP3: No. A10, 11, 17, and 21. M: 100 bp DNA ladder. PCR products for NWP2 and NWP3 were 283 bp and 549 bp, respectively.

3.2 Roe development of the two *M. cephalus* cryptic species in tropical Taiwan

NWP2 is the main species cultured in the aquaculture pond sampled in tropical Taiwan, which had a gonadosomatic index (GSI 10-20%, n=15) after culturing for two years and harvesting in November. The otolith ageing analysis suggested that all the NWP2 individuals were 2+ years old (Figure 3a), coinciding with the last year for rearing. Most NWP3 had the same age as NWP2 but with only minor roe development (Ovaries<50g, GSI<0.1-3%, n=11) (Figure 4), except for the biggest NWP3, which had a GSI of 14.3 (Ovary >300g) and was identified as the only 3+ year-old (Figure 3b). The mean GSI between NWP2 and NWP3 was significantly different ($p<0.05$).

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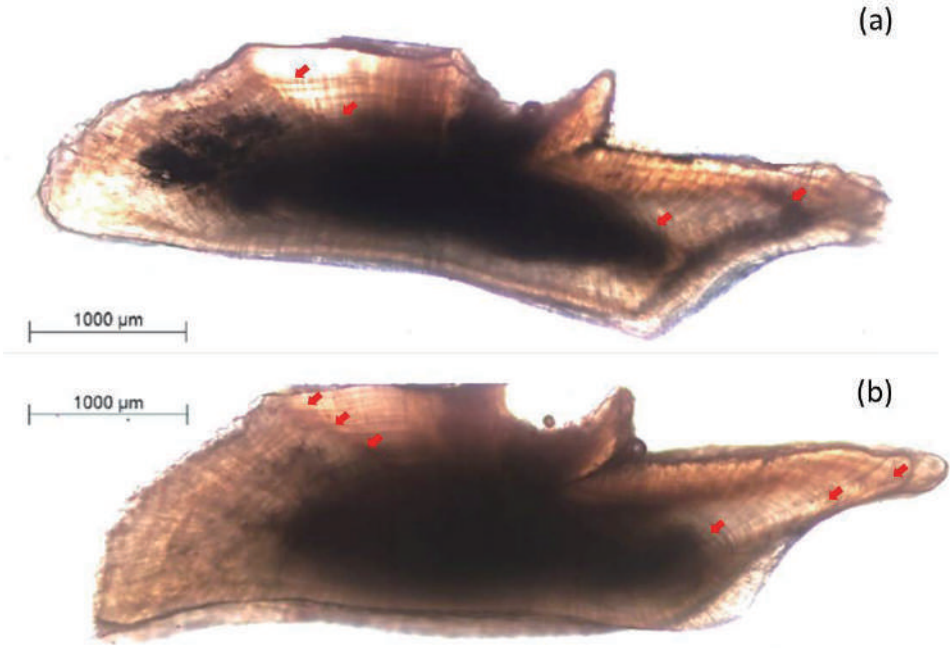

Figure 3. Otolith annual rings of *M. cephalus* visualized by transverse sections of the left sagitta (indicated by a red arrow) for 2+ (a, no. A02) and 3+ (b, no. A25) year-old samples.

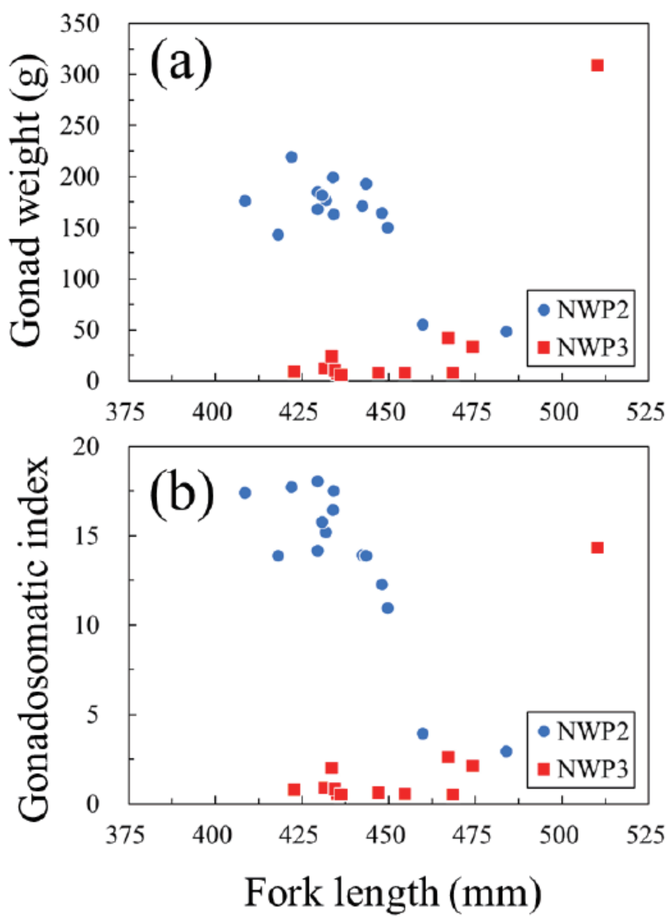

Figure 4. Change in gonad weight (a) and GSI (b) by fork length of *M. cephalus* NWP2 and NWP3 in a tropical aquaculture pond.

3.3 Somatic growth of cultured *M. cephalus* cryptic species in tropical Taiwan

All the fish sampled, both NWP2 and NWP3 could grow to around 408.5-483.9 mm in fork length (450-560 mm in total length) and 930-1,630 g in weight after culturing for two years (Figure 5). The mean fork length and body weight were not significantly different between NWP2 and NWP3 (both P>0.05). The relative growth between fork length and body weight for each species was well fitted by the power function and showed no significant difference (P>0.05), $y=0.00001x^{3.00}$ (R^2=0.74) for NWP2 and $y=0.000002x^{3.33}$ (R^2=0.96) for NWP3, although with only limited length ranges (Figure 5). There was also an extremely large 3+ year-old NWP3 with 510.28 mm of FL and 2,155 g of BW, which was obviously different from other 2+ year-old individuals.

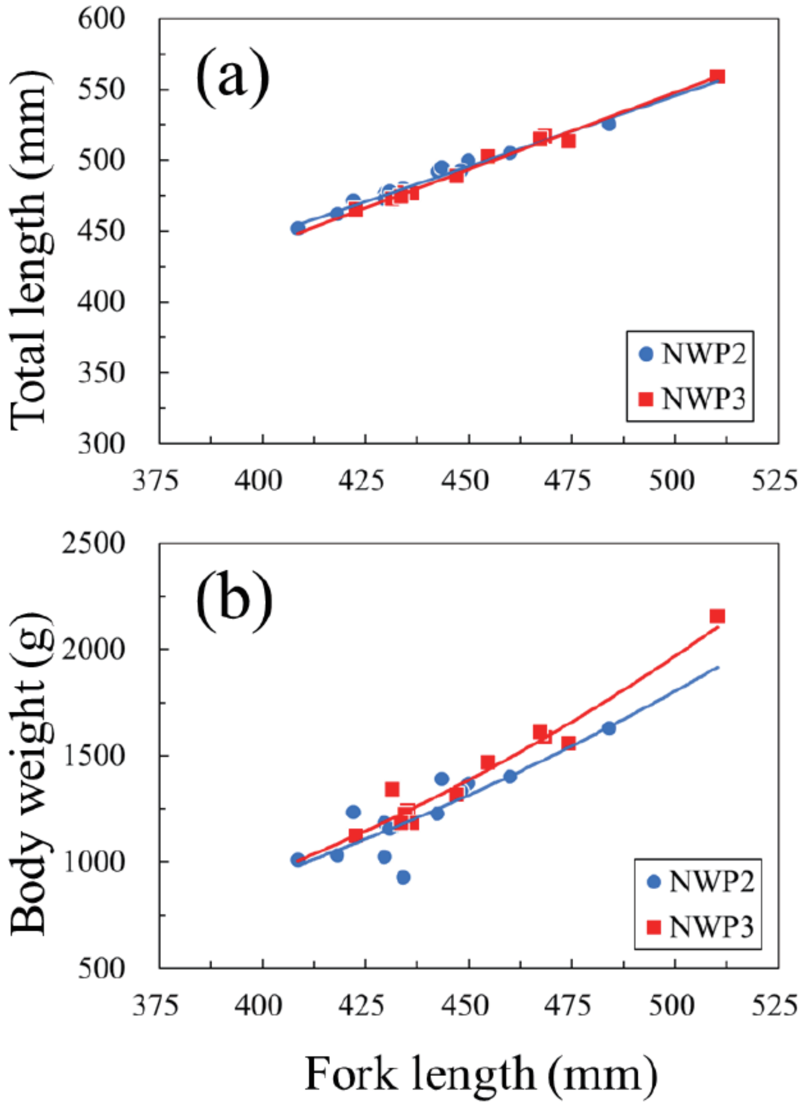

Figure 5. Change in total length (a) and body weight (b) by fork length of *M. cephalus* NWP2 and NWP3 in a tropical aquaculture pond.

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4 DISCUSSION

A previous study indicated that *M. cephalus* fished on the spawning grounds located in the Taiwan Strait belong to only one species (NWP1, Shen et al., 2015). However, the fingerlings harvested from estuaries for aquaculture use in Taiwan consist of a mixture of three different cryptic species (Ke et al., 2009; Shen et al., 2015). NWP1 and NWP2 were discovered to be the most prevalent in aquaculture ponds in both Northern and Central Taiwan with only few NWP3 found in Central Taiwan (Chien et al., 2016). However, NWP2 and NWP3 were the main species collected in a tropical fish pond in Taiwan. The results demonstrated that the mullet species composition from the pond sampled were similar to the species composition found in the tropical Kaoping River estuary where NWP2 and NWP3 were also the dominant mullet species with only few NWP1 (Shen et al., 2015). Newly recruited NWP3 larvae were only found in the tropical Baoli Creek estuary in Southern Taiwan but not in the subtropical Gonshitian Creek in Northern Taiwan during recruitment season (Shen et al., 2015). The aquaculture pond investigated in this study is located near Baoli Creek and the fingerlings used for aquaculture were likely collected from the local areas.

Considering the physiological requirements of these cryptic species may improve aquaculture practices by decreasing fingerling mortality and get better growth conditions in aquaculture ponds since their distributions presumably reflect temperature preferences (Shen et al., 2011). NWP1 was found to migrate southward with the 19-21°C front of the CCC to find appropriate sea surface temperature for spawning during December, and was only observed in the Taiwan Strait and Northeastern Taiwan where mixing of the warm and cold waters of the Kuroshio and the CCC occurs (Shyu & Lee, 1986; Hsu et al., 2007). The distribution range for NWP3 seems to follow the warm waters of the South China Current (Shen et al., 2011), and is mostly present in tropical areas (also found in tropical New Caledonia and Fiji in the Southern Hemisphere) (Durand et al., 2012; Whitfield et al., 2012; Durand & Borsa, 2015; Shen et al., 2022). The distribution range of NWP2 fits the circulation of the Kuroshio Current in the NW Pacific (Shen et al., 2011), a relatively warm ocean current with an annual average sea-surface temperature of about 24 °C (between 23-26°C). Therefore, as pointed out by Shen et al. (2011), NWP1 is more temperate adapted than the other two cryptic species and might not adapt well to the higher temperatures in tropical and subtropical aquaculture ponds as NWP3, and was not found in subtropical Northern Taiwan. In the Baoli Creek estuary of Southern Taiwan, no NWP1 was observed among juveniles sampled from December to March, while NWP2 (51.9-95.98%) and NWP3 (4.2-24.6%) were abundant (Shen et al., 2015). In the Gonshitian Creek estuary in Northern Taiwan, no NWP3 was observed among juveniles sampled during the recruitment season (from December to March) with only NWP2 present during December to February. Subsequently, there was a clear abundance shift with NWP1 in March as all juveniles collected during this month belong to the NWP1 species (Shen et al., 2015). If the aquaculture pond in Northern Taiwan obtained the fingerlings from subtropical estuaries after March, there might have been more chances to culture NWP1 as found in the aquaculture ponds in subtropical Central and Northern Taiwan (Chien et al., 2016; 2017), which received the mullet fingerlings collected from northern estuaries. The culture of *M. cephalus* in another tropical aquaculture pond (Tainan) but with fingerlings from subtropical Taipei County also showed the same species composition as the subtropical aquaculture pond of Northern Taiwan with a higher ratio of temperate NWP1 (Chien et al., 2017). Because NWP3 were largely found in tropical Taiwan, global warming may expand their distribution range northward to Central Taiwan; in addition, there was a northward movement recorded for temperate species NWP1 (Hung & Shaw, 2006).

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Since the value of "grey gold" relies on the size of the mullet roe, the roe development of different cryptic species is critical for local aquaculture. Sometimes fishermen buy flathead mullet fingerlings from local suppliers and sometimes they buy them from other cities; in response, the species composition and origins of fingerlings affect the species composition in aquaculture ponds and roe development after being cultured for 2-4 years. NPW1, NPW2, and NWP3 in the wild have similar length-weight growth equations (Figure 6). The somatic growth conditions of the cultured *M. cephalus* in Checheng were determined to be superior to that of the wild *M. cephalus* from feeding grounds by likelihood ratio test.(both species P<0.01), albeit with only limited length range data (Figure 6). This may suggest that the differences in food supplies and water temperatures could dictate the growth and development of roes among wild cryptic species and their counter parts in aquaculture ponds. Although the three cryptic species shared a common growth pattern, their roe development varied in different water temperatures, salinity, and age (Shen et al., 2015; Chien et al., 2016; 2017).

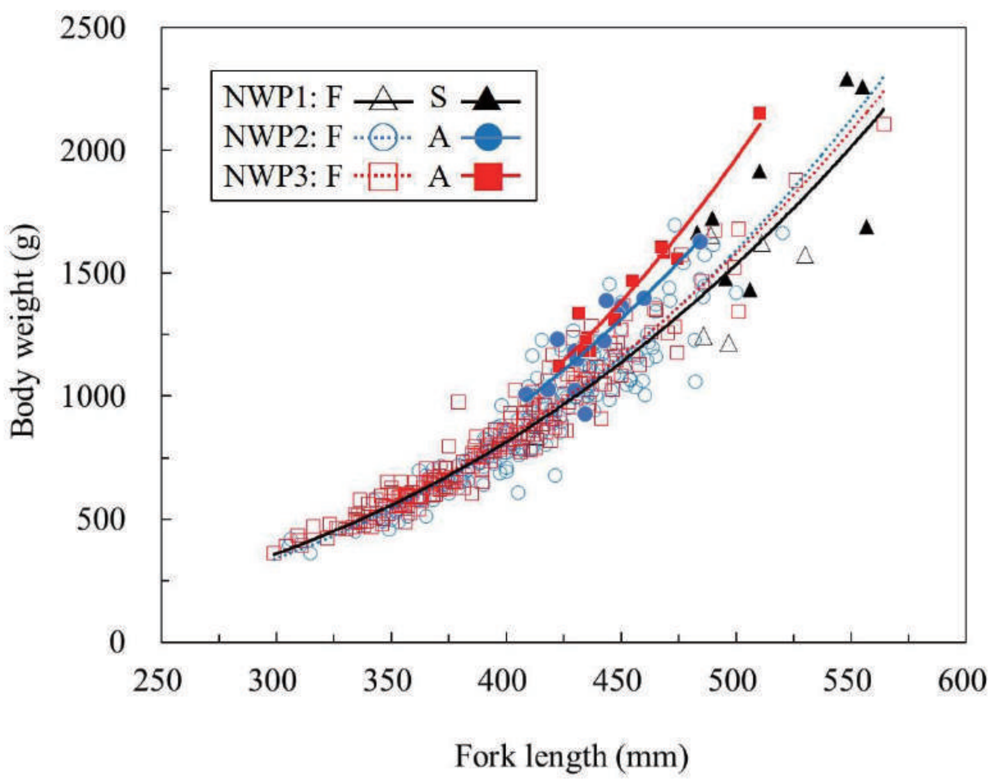

Figure 6. Growth curves comparing the cultured (A) *M. cephalus* NWP2 (solid blue (F), cycle) and NWP3 (solid red square) in this study and the wild *M. cephalus* cryptic species (NWP1, NWP2 and NWP3) from spawning ground (S) and feeding ground recalculated from Shen et al. (2015).

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In Table 1, which shows studies from various localities, the GSI for wild NWP1 in the spawning grounds may attain around 20% (17-24.6) at 423-623 mm in fork length and 1,437-2,293g in BW; while in aquaculture ponds in subtropical Taiwan, NWP1 may grow over 3,000 g but with a lower GSI compared to the spawning grounds even though they had been cultured for four years (Chien et al., 2017). The GSI for NWP2 and NWP3 in the brackish tropical feeding grounds were <10% and <19.4%, respectively, at a range between 300-500 mm in FL (Shen et al., 2015); while in aquaculture ponds in tropical Taiwan, both NWP2 and NWP3 can grow to around 408.5-483.9 mm in fork length with 930-1,630 g in weight after being cultured for two years with a GSI of 15% for NWP2 and limited development for NWP3. This means that the ovary development in tropical aquaculture ponds is slower for NWP3 during the first two years (only around 15 g compared to 160 g in average for NWP2). The extremely large individual of the NWP3 species in the aquaculture pond (Ovary>300g) and Kaoping River estuary suggests that NWP3 might also have aquaculture potential as found by Chien et al. (2018), and that NWP2 and NWP3 may attain 1,500-3,500 g with most ovaries over 200 g (range 200- 450 g) and 2,000-4,000g with most ovaries over 200g (range 200-500g), respectively, after being cultured for at least three years. After comparing the relevant results, we verified both hypotheses of our study, concluding that even wild populations have experienced temporal variations for more than 10 years while spatial genetic structures haven't changed for *M. cephalus* cryptic species. NWP2 and NWP3 were still distributed largely in tropical Taiwan waters, while NWP1 and NWP2 were only found in subtropical waters, with few NWP3 and NWP1 found in subtropical waters of Central Taiwan. The temperate NWP1 cultured in subtropical and tropical waters or NWP2 and NWP3 cultured in subtropical and tropical waters may continue to grow but with varied gonad development levels, which could indicate the plasticity of their gene expressions for adaptation, since adaptive phenotypes can evolve via different genetic mechanisms under changing environments (Bernatchez, 2016). Our extremely large NWP3 in the aquaculture pond was a 3+ year-old individual (according to otolith ageing). This was similar to our finding that 3+ year-old NWP3 cultured in Central Taiwan (Taichung) could attain 2,000-4,000 g (Chien et al., 2017). The maximum standard length and age recorded for wild *M. cephalus* was 100 cm (Ben-Tuvia, 1986) and 16 years (Thomson, 1963), respectively. The estimated growth equations for different cryptic species of cultured *M. cephalus* in this study were just a small part of their lifespan.

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Table 1. Summary of sampling location and year, identified species, sample size (n), age, and ranges in fork length (FL)/total length (TL), body weight (BW) and gonadosomatic index (GSI) of the cryptic species from the aquaculture pond in this study and other relevant references. T means tropical; TT means tropical fingerlings cultured in tropical areas; SS means subtropical fingerlings cultured in subtropical areas; ST means subtropical fingerlings cultured in tropical areas.

Location	Year	Identified Species	n	Age	FL (mm)/TL (mm)	BW (g)	GSI	References cited
Kaohsiung offshore (Spawning ground for NWP1), T	2005	NWP1	44	-	423.0-555.0	1,918.6-2,293.6	20.0-21.4	Shen et al. 2015
	2006	NWP1	50	-	421.4-533.7	1,481.0-1,727.0	17.2-24.6	
	2007	NWP1	57	-	459.6-623.9	1,437.0-1,692.0	17.0-19.1	
Kaoping River estuary (Feeding ground), T	2008-2009	NWP1	7	-	413.0-530.0	836.6-1,656.0	>0.1-20.1	Shen et al. 2015
		NWP2	184	-	305.4-520.0	363.0-1,699.2	>0.1-10.1	
		NWP3	209	-	299.0-564.2	361.4-2,108.6	>0.1-19.4	
Hsinchu country (Aquaculture pond), SS	2013	NWP1	44	2	-	-	>0.1->15	Chien et al. 2016
		NWP2	46	3	-	-	>0.1->15	
Ilan country (Aquaculture pond), SS	2013	NWP1	2	4	-	-	<5	
		NWP2	42	4	-	-	>0.1->15	
Changhua County (Aquaculture pond), SS	2013	NWP1	22	3	-	-	<5	
		NWP2	23	3	-	-	>0.1->15	
		NWP3	2	3	-	-	<5	
Taipei County (Aquaculture pond), SS	2013	NWP1	36	3	-	-	>0-15	
		NWP2	10	3	-	-	>0.1->15	
Changhua county (Aquaculture pond), SS	2013	NWP1	22	3	-	3,285.8±590.1	most<5	Chien et al. 2017
		NWP2	23	3	-	2,169.3±464.9	<15	
Taipei county (Aquaculture pond), SS	2013	NWP1	36	3	-	2,512.7±303.9	most<5	
		NWP2	10	3	-	2,365.5±360.3	<20	
Taipei county (Aquaculture pond), ST	2015	NWP1	85	4	-	3,734.9±599.2	most<10; few 10-15	
		NWP2	17	4	-	2,790.6±599.6	9<->15	
Taichung County (Aquaculture pond), ST	2015	NWP2	136	3	-	1,500-3,000 (Range)	>0.1->15	Chien et al. 2018
		NWP3	35	3	-	2,000-4,000 (Range)	>0.1->15	
Checheng, Pingtung (Aquaculture pond), TT	2019	NWP2	15	2	408.5-483.9/ 451.5-525.3	835-1,630	2.9-18	This study
		NWP3	12	2/3	422.7-510.2/ 465.0-558.9	1,185-2,155	>0.5-14.3	

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5 CONCLUSIONS

This study's aquaculture pond in Checheng, in tropical Taiwan, cultured two mullet cryptic species, coinciding with their preference for higher temperatures compared to NWP1. Furthermore, NWP2 and NWP3 attained sizes of around 408.5-483.9 mm in fork length and 930-1,630 g in body weight after rearing for two years, but with limited roe development for NWP3. In a mixed species aquaculture pond, as the ones for *M. cephalus* in Taiwan, it is recommended to collect fingerlings from nearby waters and cultured for at least three years to achieve better roe development for both species. *M. cephalus* cryptic species have maintained their spatial genetic structures over the course of more than a decade. The varied gonadal development levels for the same cryptic species cultured in different climate zones suggested their gene expression plasticity for adaptation.

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