Chapter 3

Application of Karyotype and Genetic Characterization Analyses for Hybrid Breeding of *Epinephelus* Groupers

Mei-Chen Tseng and Kuan-Wei Shih

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80414

Abstract

In this study, karyotypes and Cyt *b* gene sequences of seven different species of grouper including *Plectropomus leopardus*, *Epinephelus coioides*, *E. flavocaeruleus*, *E. fuscoguttatus*, *E. lanceolatus*, *E. polyphekadion*, and *E. tukula* were examined. All chromosome numbers from seven groupers were 2n = 48 with a high number of telocentric chromosomes (38–48) and fundamental arm numbers (FNs) (48–54). The mitochondrial Cyt *b* gene was used to establish the barcodes of seven groupers and analyze phylogenetic relationships among these species. We discovered that *Epinephelus* groupers should be classified as monophyly. The minimum genetic distance expressed between *E. coioides* and *E. tukula* was 0.1276. From results of the cytogenetic and molecular analyses, it was demonstrated that *Plectropomus* is a relatively primitive genus of grouper, while *Epinephelus* is a more-modern derived genus. Results also showed that *E. coioides* and *E. tukula* have similar genetic characters and karyotypes, and should be foremost considered for artificial hybridization strategies. Furthermore, information on karyotypes of species within the *Epinephelus* is still insufficient, and further elucidation of karyotypes of *Epinephelus* will be a great help to future genetic breeding research.

Keywords: barcode, cytochrome *b*, cytogenetic, genetic distance, hybridization

1. Introduction

Epinephelus groupers (Perciformes, Serranidae) are widely distributed in tropical and subtropical waters [1] and comprise 89 species (valid names) in marine habitats worldwide [2]. Most known grouper species are in the Indian-Pacific Ocean, 11 species along the West Atlantic coast, nine species in the East Atlantic Ocean and Mediterranean, and eight species in the eastern Pacific Ocean. Only a few groupers are distributed across different oceans [1]. Forty-one species of groupers in total were found in coastal waters of Taiwan [3].

Grouper is an important aquatic product in the world. In addition to abundant grouper caught at sea, the artificial breeding grouper is also a major aquatic product in the fishery trade. In the past, most grouper fry were from Southeast Asian countries such as the Philippines, Indonesia, and Thailand. However, survival rates markedly decreased due to catching and transportation. Nowadays, breeding techniques have been completely established for major commercial groupers, and so most grouper fry are bought from artificial breeding farms. Currently, *Epinephelus akaara*, *E. areolatus*, *E. awoara*, *E. bleekeri*, *E. bruneus*, *E. fuscoguttatus*, *E. lanceolatus*, *E. septemfasciatus*, *E. tauvina*, *E. coioides*, and *E. malabaricus* can be artificially reared and bred, especially *E. malabaricus*, which is the most successful case. Groupers have similar external morphologies, and their body color characteristics are not stable. Juveniles and adult fishes may show completely different color patterns. Therefore, it is often impossible to effectively distinguish species with similar morphologies in the adult stage [1, 4, 5]. As to their mating systems, incorrect identification of parents and progeny in rearing and breeding farms may cause artificial full-breeding plans and hybridization strategies to fail; moreover, this will result in significant fishery losses [1, 5, 6].

Traditionally, grouper species were classified using morphological and skeletal features [1, 7–9]. In the past two decades, molecular genetic technology has been dramatically developed and is now widely used in taxonomic and systematics studies. As Ref. [5] analyzed 42 species of grouper including three genera (*Epinephelus*, *Cephalopholis*, and *Mycteroperca*) using partial 16S ribosomal (r)DNA sequences. Results of that phylogenetic study revealed that both genera *Epinephelus* and *Mycteroperca* belong to the same clade, and it was inferred that Serranidae comprised a paraphyletic group.

Nowadays ichthyologists also use variable staining methods to obtain cytogenetic information of fish [10, 11]. According to previous studies, the number of chromosomes in groupers are 2n = 48, most of which are telocentric chromosomes, and fundamental numbers range 48–62 [12]. Some reports on the cytogenetics of grouper indicated that silver-binding nucleolar organizing regions (Ag-NORs) are highly conserved on the chromosome 24, but variations occur in the location between different groupers [13–18]. It is generally believed that such variations may be caused by an inversion of the arms during chromosome evolution. To study an evolutionary model of chromosomes and identify species, staining techniques were used often to analyze the karyotype and cytogenetics of groupers.

More than ten groupers have been successfully cultivated in Taiwan. However, most groupers have similar external morphologies, and their color patterns are quite unstable. Often grouper in different life stages exhibit inconsistent color distributions that resulted in the species identification of grouper fry being controversial or confusing [1, 5]. In the aquaculture industry, misidentification frequently occurs in different growth stages of groupers, and this can cause serious problems, such as chaos of market prices, interspecific ecological competition, and breeding strategy failures.

It is important to understand the karyotype and phylogeny of cultured grouper for a successful strategy of genetic breeding. That is when studying hybridization strategies of groupers, selecting similar karyotypes and closely related species for the parents may

http://dx.doi.org/10.5772/intechopen.80414

result in relatively higher success potential for hybridization. Therefore, the establishment of grouper karyotype and barcode data in this study will provide more-perfect genetic bases for species identification to improve possibilities for genetic breeding. The present study analyzed the mitochondrial cytochrome (Cyt) *b* gene sequences and chromosomal characters of seven cultured groupers in Taiwan. These results will provide farmers with more genetic information of groupers to develop useful breeding strategies for hybridization in the future.

2. Materials and methods

2.1. Sampling

Seven groupers, *Epinephalus lanceolatus*, *E. tukula*, *E. flavocaeruleus*, *E. polyphekadion*, *E. fuscoguttatus*, *E. coioides*, and *Plectropomus leopardus*, were collected from fish markets in Tungkang, southern Taiwan (**Figure 1**) for chromosome preparation and DNA sequence analysis. A piece of muscle tissue from each specimen was preserved in 95% ethanol (EtOH) and stored at the Fish Biology Lab in National Pingtung University of Science and Technology. Seven species were used for the karyotype analysis and Cyt *b* gene sequencing.

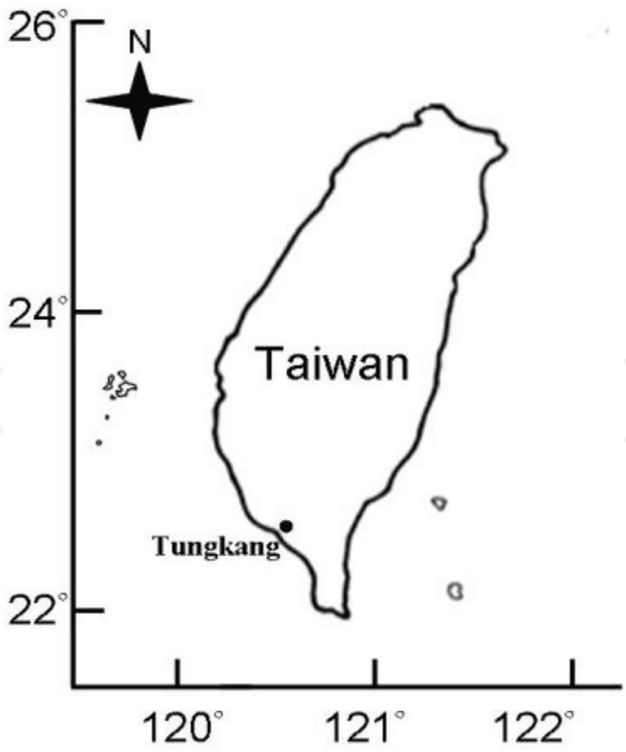

Figure 1. Sampling location of groupers.

2.2. Chromosomal preparation and karyotype analyses of groupers

The cell culture solution contained Eagle's minimal essential medium (MEM) with 15% fetal bovine serum and 0.0001% colchicine, followed by filter-sterilization (0.45 μm). Kidney tissue was cut and placed in the cell culture solution. The solution tubes were placed on a rotary shaker (100 rpm) and then incubated at room temperature for 2 h to allow cells to remain in the metaphase of the cell cycle. The cell culture solution was centrifuged at 3000 rpm for 5 min, and the supernatant was discarded. KCl (at 0.075 M) was added and allowed to sit at room temperature for 30 min. After centrifugation at 3000 rpm for 5 min, the supernatant was discarded, and a freshly prepared fixative solution (methanol: acetic acid = 3:1) was added at room temperature for 15 min. The mixture was centrifuged at 3000 rpm for 5 min, the supernatant was discarded, and this step was repeated two or three times. The cell suspension was dropped onto a heated glass slide and air-dried. After the slide had been stained with 5% Giemsa dye for 10 min, it was rinsed with water and air-dried. The slide was mounted and observed by microscopy.

In addition, some fresh chromosome slides were stained with $AgNO_3$. Two drops of 2% (w/v) gelatin and four drops of a 50% $AgNO_3$ solution were mixed and then dropped onto a slide with a cover glass. These slides were incubated at 70°C until they presented a yellowish-brown color. The slides were gently rinsed with double-distilled (dd)H_2O. After being air-dried at room temperature, the slides were mounted with gum arabic [19]. Chromosomes were observed with an optical microscope (Leica Microsystems, Wetzlar, Germany) (at 1000× with an oil lens). Digital images of the chromosomes were recorded and analyzed with a chromosome band analytical system (BandView 5.5, Applied Spectral Imaging, Migdal HaEmek, Israel). Chromosomes stained with Giemsa were classified into four groups, metacentric (m), submetacentric (sm), subtelocentric (st), and telocentric (t), according to the system described by [20]. Locations of chromosomes determined by $AgNO_3$ staining were observed and marked on photos.

2.3. DNA isolation

Approximately 100 mg of muscle tissue from each specimen was put into an Eppendorf tube. Before DNA purification, the tube was placed in a 60°C oven for 10 min to evaporate the EtOH. Genomic DNA was isolated using a Gentra Puregene Core kit A (Qiagen, Venlo, the Netherlands), and the purified DNA specimen was dissolved in TE buffer (1 M Tris–HCl at pH 8.0 and 0.2 mL EDTA, 0.5 M). DNA concentrations were estimated using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at an absorbance of 260 nm. The purity of DNA preparations was checked by the ratio of absorbances at 260 and 280 nm ($A_{260}/A_{280} \geq 1.8$). DNA stock solutions were stored in a − 20°C freezer.

2.4. Cyt *b* gene sequencing and analysis

In total, 50 μL of reactant of a polymerase chain reaction (PCR) contained 5 ng genomic DNA, 10 pmol each of the forward and reverse primers, 4 μL 2.5 mM dNTP, 0.2 μL 25 mM $MgCl_2$, 1 U *Taq* polymerase, and 5 μL 10× buffer, with ddH_2O added to 50 μL. The forward and reverse primers of the Cyt *b* gene were FOR (5′-CGAACGTTGATATGAAAAACCATCGTTG-3′) and UnvH (5′-ATCTTCGGTTTACAAGAC CGGTG-3′), respectively [6]. The Cyt *b* gene was

amplified using a PCR machine (BIO-RAD MJ Mini Gradient Thermal Cycler, Conmall Biotechnology, Singapore) with initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; with a final extension of 72°C for 10 min. The reaction was cooled down to 25°C for 10 min. PCR products of the Cyt *b* gene were checked using 1% agarose gel electrophoresis and then stained with ethidium bromide (EtBr; 0.5 mg/mL). Target DNA fragments were eluted with a DNA Clean/Extraction kit (GeneMark, Taichung, Taiwan). Sizes of the purified DNA fragments were checked and then stored in a −20°C freezer. DNA fragments were directly sequenced on an Applied Biosystems (ABI, Foster City, CA, USA) automated ABI3730x1 DNA sequencer using a Bigdye sequencing kit (Perkin-Elmer, Wellesley, MA, USA). FOR or UnvH primers were used in the sequencing reaction, and the PCR cycle parameters for sequencing were 35 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C.

In total, seven Cyt *b* sequences were obtained in this study. Homologous sequences were aligned using ClustalW [21] and then manually checked. Interspecific genetic distances were analyzed using the Kimura-2-parameter (K2P) model [22], and numbers of different nucleotides were calculated with MEGA software [23]. The best-fitting models of DNA substitution were determined using the lowest Bayesian Information Criterion (BIC) scores [24]. The phylogenetic trees of Cyt *b* sequences were constructed using the Neighbor-joining (NJ) [25] and Maximum-likelihood (ML) methods [26]. Cluster confidence levels of Cyt *b* were assessed using a bootstrap analysis with 1000 replications [27].

3. Results

3.1. Karyotype analyses

In cytogenetic studies, Giemsa staining of seven groupers indicated that the diploid number of these species was 2n = 48. The karyotypic formulae were 2 sm + 46 t for *E. coioides*, *E. fuscoguttatus*, and *E. tukula*; 6 sm + 4 st + 38 t for *E. lanceolatus*; 2 st + 46 t for *E. flavocaeruleus*; 6 sm + 42 t for *E. polyphekadion*; and 48 t for *P. leopardus*. All of those specimens had a high number of telocentric chromosomes (38–48) and fundamental arm numbers (FNs) that ranged 48–54 (**Figure 2**, **Table 1**).

In Ag-NO_3 staining, four *Epinephelus* species (*E. coioides*, *E. fuscoguttatus*, *E. tukula*, and *E. lanceolatus*) and *P. leopardus* were completed. *Epinephelus coioides*, *E. fuscoguttatus*, and *E. tukula* had one pair of Ag-NORs located on the short arm of the sm chromosome; *E. lanceolatus* had two pairs of Ag-NORs located on the short arm of the sm chromosome; and *P. leopardus* had one pair of Ag-NORs, located near the centromere of larger telocentric chromosomes (**Figure 3**).

3.2. Cyt *b* sequence analysis

All Cyt *b* gene sequences from seven groupers were 1141 bp for *E. lanceolatus*, *E. tukula*, *E. flavocaeruleus*, *E. polyphekadion*, *E. fuscoguttatus*, *E. coioides*, and *P. leopardus*. Percentages of nucleotide compositions did not significantly differ among these *Epinephelus* species, as the A + T ratios were in the range of 52.1% (*E. flavocaeruleus*) - 56.7% (*E. polyphekadion*). Interspecific

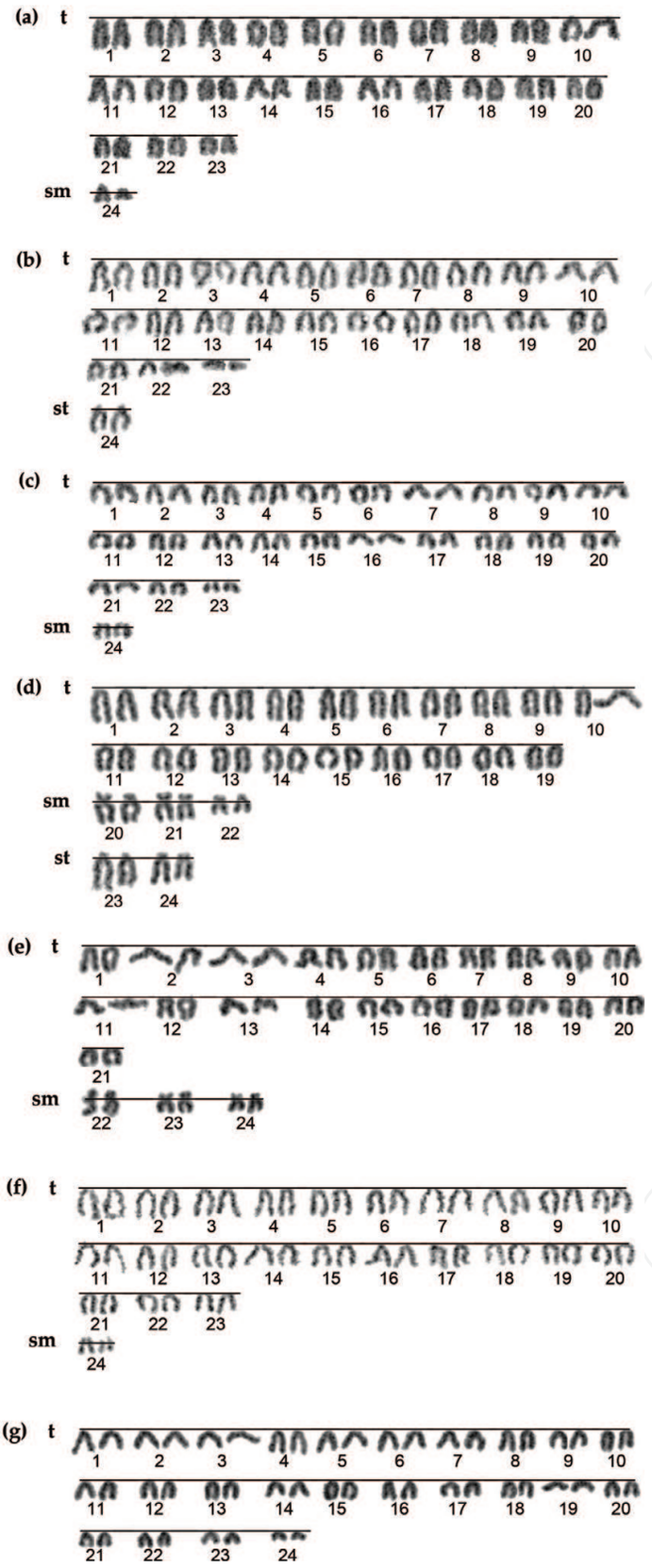

Figure 2. Karyotype analyses of seven groupers: (a) *Epinephelus coioides*; (b) *E. flavocaeruleus*; (c) *E. fuscoguttatus*; (d) *E. lanceolatus*; (e) *E. polyphekadion*; (f) *E. tukula*; and (g) *Plectropomus leopardus*.

Species	2*n*	FN	Formulae	Reference
E. adscensionis	48	48	48 a	[28]
E. akaara	48	48	5 st + 43 a	[40]
E. alexandrinus	48	48	48 a	[15]
E. awoara	48	48	48 a	[13]
E. bruneus	48	54	2 m + 4 sm + 42 a	[46]
E. caninus	48	48	48 a	[16]
E. coioides	48	50	2 sm + 46 a 2 sm + 46 t	[12] Present study
E. diacanthus	48	50	2 sm + 46 a	[41]
E. fario	48	62	4 m + 6 sm + 4 st + 34 a	[42]
E. fasciatomaculosus	48	48	48 a	[43]
E. fasciatus	48	48	48 a	[43]
E. faveatus	48	50	2 m + 46 a	[48]
E. flavocaeruleus	48	48	2 st + 46 t	Present study
E. fuscoguttatus	48	50	2 sm + 46 t	Present study
E. guaza	48	48	48 a	[15]
E. guttatus	48	48	48 a	[15]
E. lanceolatus	48	54	6 sm + 4 st + 38 t	Present study
E. malabaricus	48	48	48 a	[44]
E. marginatus	48	48	48 a	[18]
E. merra	48	62	4 m + 6 sm + 4 st + 34 a	[42]
E. moara	48	48	48 a 4 sm + 44 a	[45] [46]
E. polyphekadion	48	54	6 sm + 42 t	Present study
E. sexfasciatus	48	50	2 sm + 46 a	[47]
E. tauvina	48	50	2 sm + 46 a	[16]
E. tukula	48	50	2 sm + 46 t	Present study
P. leopardus	48	48	48 t	Present study

E., Epinephelus; *P., Plectropomus*; 2*n*, diploid number; FN, fundamental number; metacentric (m), submetacentric (sm), subtelocentric (st), and telocentric (t), according to the system described in Ref. [20].

Table 1. Karyotype data of the Epinephelinae.

p-distances and K2P genetic distances ranged 0.1149 and 0.1284 (*E. tukula vs*. *E. coioides*) to 0.1814 and 0.2138 (*E. flavocaeruleus vs*. *E. polyphekadion*) (**Table 2**). The best model of nucleotide evolution was estimated to be the TN93 + G + I model with BIC = 9065.099. The NJ and ML analyses showed that *E. tukula* and *E. coioides* had a close phylogenetic relationship with extremely high bootstrap support (**Figure 4**). This result agreed with the hypothesis that *Epinephelus* is a monophyletic group.

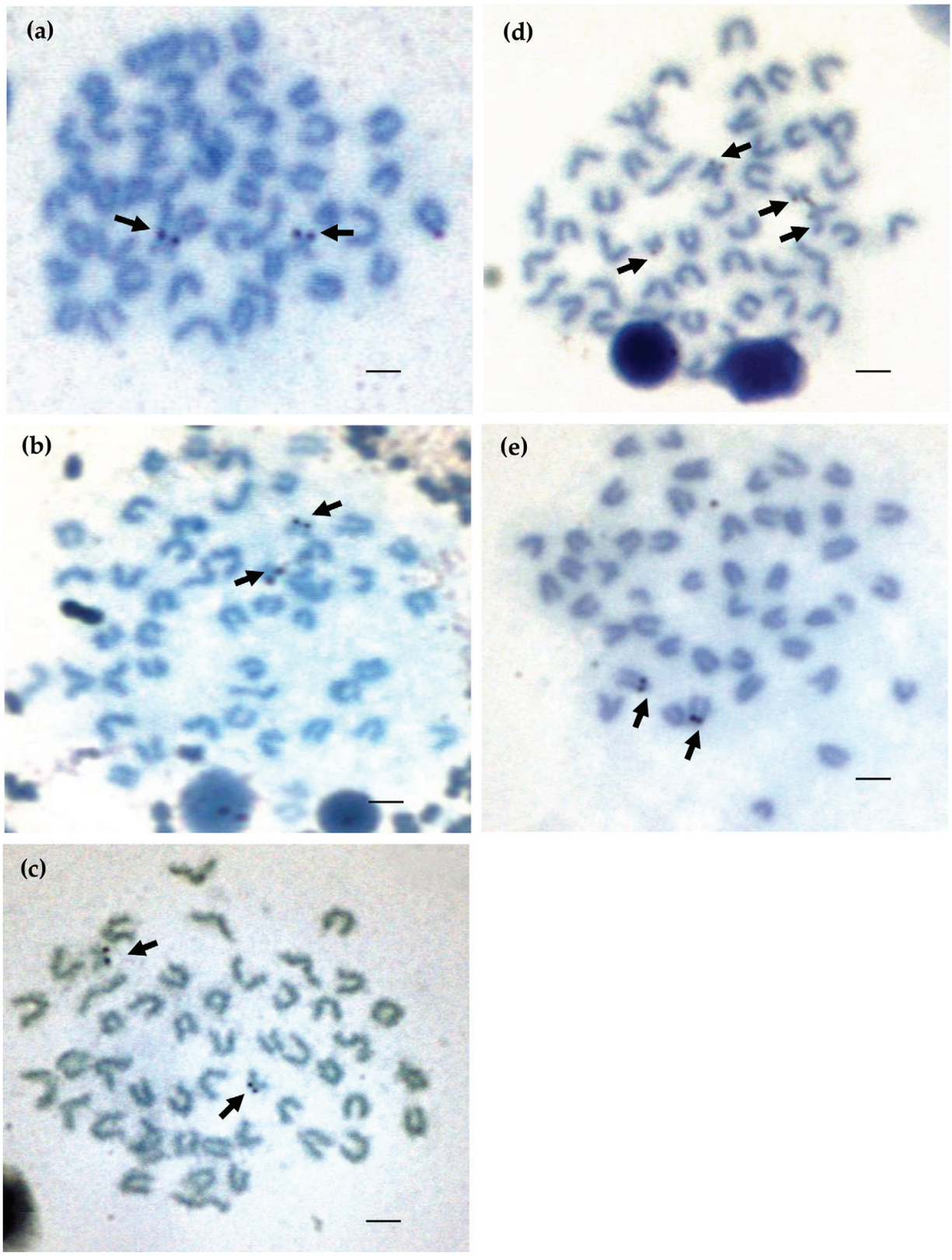

Figure 3. Silver-binding nucleolar organizing regions (Ag-NORs) results from five groupers: (a) *Epinephelus coioides*; (b) *E. fuscoguttatus*; (c) *E. tukula*; (d) *E. lanceolatus*; and (e) *Plectropomus leopardus*. The arrows indicate Ag-NORs. The bar equals 5 μm.

Code	Species name	1	2	3	4	5	6	7
1	*E. lanceolatus*	—	0.1422	0.1649	0.1474	0.1430	0.1333	0.2344
2	*E. tukula*	0.1635	—	0.1658	0.1684	0.1360	0.1149	0.2186
3	*E. flavocaeruleus*	0.1908	0.1934	—	0.1814	0.1578	0.1604	0.2272
4	*E. polyphekadion*	0.1690	0.1994	0.2138	—	0.1516	0.1595	0.2237
5	*E. fuscoguttatus*	0.1638	0.1561	0.1817	0.1751	—	0.1350	0.2123
6	*E. coioides*	0.1510	0.1284	0.1853	0.1858	0.1436	—	0.2307
7	*P. leopardus*	0.2859	0.2617	0.2738	0.2699	0.2529	0.2803	—

Table 2. *p*-distance genetic distances (above the diagonal) and Kimura 2-parameter distances (below the diagonal) of cytochrome *b* gene sequences among *Epinephelus* groupers and the outgroup *Plectropomus leopardus*.

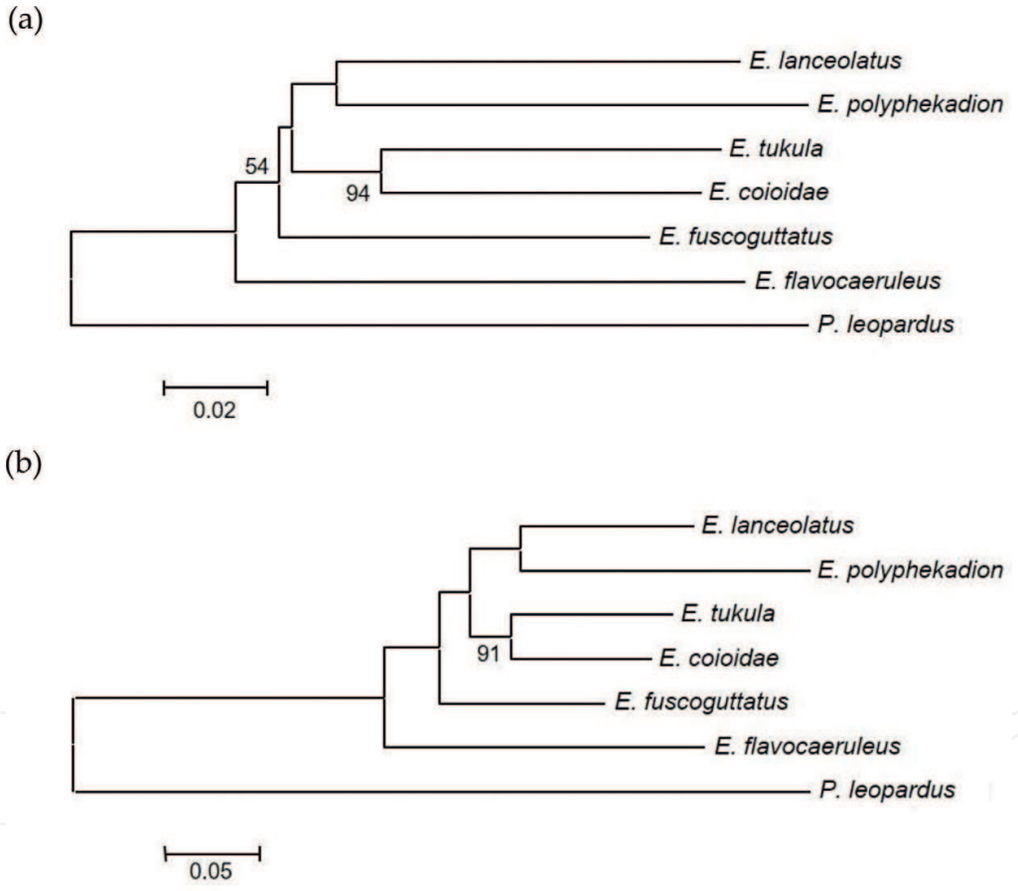

Figure 4. (a) The Neighbor-joining and (b) the Maximum-likelihood trees among *Epinephelus* species based on the cytochrome *b* gene analysis.

4. Discussion

In this study, Epinephelinae fish (*E. lanceolatus*, *E. tukula*, *E. flavocaeruleus*, *E. polyphekadion*, *E. fuscoguttatus*, *E. coioides*, and *P. leopardus*) showed a common synapomorphic character of

chromosomal number, 2n = 48, and high numbers of telocentric chromosomes (38–48). By sorting out the cytogenetic information of 23 Epinephelinae species, it was found that chromosomal numbers of these groupers were 48, showing highly conserved characteristics, and FNs ranged 48–62, with more than half of these groupers exhibiting FN = 48 characteristics (**Table 1**), in accordance with conservative chromosomal morphological features described in Ref. [28]. In the other hand, variations in FNs are mainly caused by chromosomal rearrangements and play important roles in the speciation process [29].

In cytogenetic studies, karyotypes, FNs, Ag-NORs, and C-bands were demonstrated to have interspecific specificities, and many studies used these techniques to explore interspecific evolutionary relationships [30–32]. Currently, reports related to chromosomes of *Epinephelus* groupers worldwide are only available for 23 of 89 groupers; *e.g*., karyotypes of *E. marginatus* were analyzed from three different sampling sites in the Mediterranean. Results showed chromosomal numbers of 2n = 48; conserved C-bands and Ag-NOR positions were observed on the 24th pair of chromosomes of specimens from all three samples, but those were also found on 2nd pair chromosomes of one specimen [18]. In order to confirm the above results, fluorescence in situ hybridization (FISH) was performed using 18S rDNA as a probe. Fluorescence reacted to the 2nd and 24th pairs of chromosomes confirming that a difference existed between samples. The authors reasoned that this may have been a species-specific manifestation, and further studies are required to confirm whether they can be population-specific markers.

Molecular phylogenetic analyses showed that both *Plectropomus* and *Cephalopholis* are more primitive genera than *Epinephelus* [5, 6, 12, 33, 34]. In this study, the chromosomal number of *P. leopardus* was 2n = 48 t. All current cytogenetic studies of *Epinephelus* groupers have shown that few of them are not composed of 2n = 48 t. These results support 48 t being an ancestral character of Serranidae fish [12], and *Epinephelus* groupers may be a later-derived genus.

In Ref. [12] observed three types of Ag-NORs distribution pattern: type I has only one pair of Ag-NORs located in the subcentromeric region of the acrocentric (t) chromosome, *e.g*., *E. guaza*, *E. alexandrinus*, *E. caninus*, *E. fasciatomaculatus*, *E. fasciatus*, and *E. awoara*; type II has one pair of Ag-NORs located in the subcentromeric region of the t chromosome pair and an extra pair of smaller Ag-NORs located on another pair of chromosomes, as in *E. adscensionis*, *E. marginatus*, and *E. malabaricus*; and type III has only one pair of Ag-NORs located on the short arm of bi-armed chromosomes, *e.g*., *E. guttatus* and *E. coioides*. Thus, based on the available cytogenetic data on the genus *Epinephelus*, most of the NORs of groupers are located on the 24th pair of chromosome (type I), and these results are consistent with those of [18]. In this study, *E. fuscoguttatus*, *E. tukula*, and *E. lanceolatus* also belonged to type III. It is generally believed that the appearance of one pair of Ag-NORs is the ancestral character of Serranidae fish [28]. However, when Ref. [12] classified this character and compared it to data of molecular phylogenies, results were found to be irrelevant. The authors believe that the contradiction between cytogenetic and molecular phylogenetic analyses may merely be the result of insufficient data.

Hybrid breeding often produces heterosis offspring, such as offspring with a fast growth rate, strong disease resistance, or diverse morphology. For example, Liu et al. crossed different carps

to obtain hybrids with a high growth rate [35]. However, many studies have found that the success possibility and whether the offspring are fertile are related to the parental karyotypes. The parents having more-similar karyotypes can increase the success ratio of hybridization [36]. At present, completely cultured groupers mainly consist of *E. akaara*, *E. areolatus*, *E. awoara*, *E. bleekeri*, *E. bruneus*, *E. fuscoguttatus*, *E. lanceolatus*, *E. septemfasciatus*, *E. tauvina*, *E. coioides*, and *E. malabaricus*. Establishment of karyotypic data of these groupers can provide references for crossing strategies on farms. The genetic relationship and chromosome composition of hybrid progeny can also be confirmed by a karyotype test.

Species names of different groupers have always been confusing. Most groupers living coral reef areas have similar external morphologies, and their color characteristics also may change along with their living environment. Some larvae and juveniles may even have completely different color distributions from adults, such as *E. lanceolatus* which has three irregular black spots and a brilliant color as juveniles, but becomes dark brown as adults. Therefore, identifying groupers is often controversial [1, 4, 5]. For example, *E. coioides* and *E. tauvina* are very similar and difficult to distinguish in Taiwanese waters [37]. There is still much dispute over the taxonomy of groupers when using traditional morphology. Cyt *b* gene marker is of great help in identifying similar groupers or unidentifiable fry. In the future, this marker can also be used in aquaculture breeding to reduce failures and losses with artificial reproduction.

In this study, the results showed that different groupers can be identified by analyzing the Cyt *b* gene. The phylogenetic tree constructed from the Cyt *b* gene can distinguish *Epinephelus* groupers from those in the genus *Plectropomus*. However, groupers evolved as monophyletic group, the genus *Plectropomus* is a relatively primitive group in Epinephelinae.

Epinephelus lanceolatus was previously classified in the genus *Promicrops* by [38, 39], but [6] used Cyt *b* to study molecular phylogenetic relationships of six out of 28 genera in the Serranidae, suggested that *Promicrops lanceolatus* should be classified into *Epinephelus*. Phylogenetic trees constructed with the NJ and ML methods also revealed that *E. lanceolatus* has a close relationship with other *Epinephelus* groupers [6]. In addition, scientific names of seven farmed groupers have been identified to reduce confusion and controversy.

5. Conclusions

All chromosome numbers from seven groupers (*Plectropomus leopardus*, *Epinephelus coioides*, *E. flavocaeruleus*, *E. fuscoguttatus*, *E. lanceolatus*, *E. polyphekadion*, and *E. Tukula*) showed a common synapomorphic character of chromosomal number, 2n = 48. Four groupers, *E. coioides*, *E. polyphekadion*, *E. fuscoguttatus*, and *E. tukula* shared the same karyotype formula of 2 sm + 46 t. *E. coioides*, *E. fuscoguttatus*, and *E. tukula* had one pair of Ag-NORs located on the short arm of the sm chromosome. The mitochondrial Cyt *b* gene was used to analyze phylogenetic relationships among these species. We discovered that *Epinephelus* groupers should be classified as monophyly. The minimum genetic distance expressed between *E. coioides* and *E. tukula* was 0.1276. Results showed that *E. coioides* and *E. tukula* have similar genetic characters and cell karyotypes, and should be foremost considered for artificial hybridization strategies.

Information on karyotypes of species within the *Epinephelus* is still insufficient, and further elucidation of karyotypes of *Epinephelus* will be a great help to future genetic breeding research.

Acknowledgements

The authors express their gratitude to IW Shih for assistance with laboratory work, and also thank Professor TB Yen for his help on image process and data integration.

Research funding was provided to MC Tseng by the Ministry of Science and Technology, Taiwan (NSC 102-2313-B-020-002).

Conflict of interest

Both authors, Mei-Chen Tseng and Kuan-Wei Shih declare that they have no conflict of interest.

Author details

Mei-Chen Tseng* and Kuan-Wei Shih

*Address all correspondence to: mctseng@mail.npust.edu.tw

Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, Taiwan, R.O.C.

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