

Mackerel Identification by Morphological Analysis and DNA Barcoding

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Abstract. *Rastrelliger* genus are caught in large quantities in Thailand and this has led to declines in the natural populations of the fish. Attempts have been made to improve stocks through breeding programs. However, better methods of identifying fish species are required to improve the selection of breeding stock. In this research, we aimed to identify species by combining the morphological method and DNA barcoding. The results of the morphometric analysis of thirty fish samples were that there were 16 *R. brachysoma* and 14 *R. kanagurta*. The ratios of forked length to operculum depth for *R. brachysoma* and *R. kanagurta* were in the ranges of 3.6-4.1 and 4.5-5.1, respectively. The intestines of *R. brachysoma* were very long, which was apparent in the ratio of intestine length to fork length being in the range of 3.4-5.7. In contrast, the intestines of *R. kanagurta* were shorter and were only 0.8-1.9 times longer than the fork. Subsequently, all samples were identified by DNA barcoding. For a conclusive identification, samples had to have >99% similarity to reference sequences in both the GenBank and BOLD systems. Moreover, a phylogenetic tree constructed based on the *COI* sequences distinguished the fish samples into 2 major clades: *R. brachysoma* and *R. kanagurta*, which correlated with the genetic classification. For twenty-two samples, there was correlation between the morphological and genetic species assignment, but for 8 samples the morphological and genetic assignments were inconsistent. Therefore, in order to achieve accurate species identification, a combined morphometric-genetic analysis is recommended.

1 Introduction

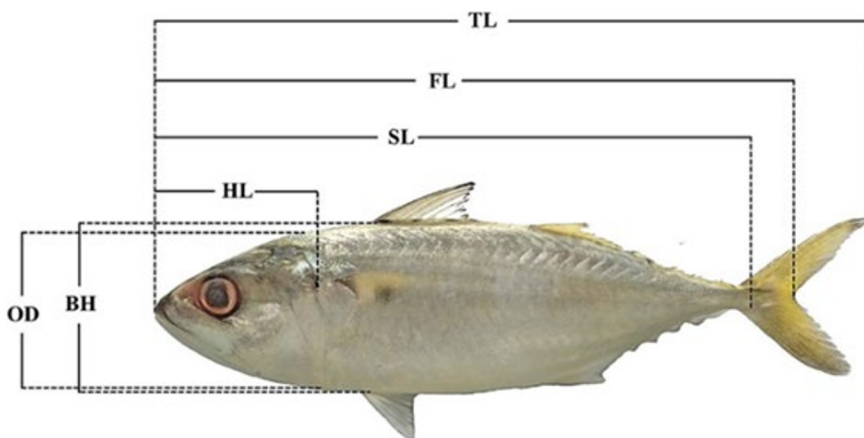
The *Rastrelliger* mackerel is a pelagic fish species, belonging to the Scombridae family. The *Rastrelliger* mackerel genus comprises three species, *R. brachysoma* (Short mackerel), *R. kanagurta* (Indian mackerel), and *R. faughni* (Island mackerel). They are widely distributed in the Indo-Pacific Ocean [1]. Mackerel is a popular species of marine food with Thai people because it is delicious, cheap, and nutrition-rich. The mackerel is therefore an important economic species in Thailand. Over the period of 2018-2022, the *Rastrelliger* mackerel, and especially *R. brachysoma* and *R. kanagurta*, was the fifth most caught marine species in Thailand. This extensive level of fishing resulted in a decrease in the natural

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populations of the fish [2-3]. In addition to overfishing, changes in the environment and natural food sources affect the natural abundance of mackerel. Effective fishery management of mackerel resources is required, and one aspect of such management is improved species identification of bloodstock. However, information to do with the classification of the three mackerel species in the *Rastrelliger* genus has not been extensively reported on. It was previously observed that the three species of mackerel differ in size and length [1]. Traditional methods for fish species identification have been carried out based on morphological characteristics [4]. However, some species are very similar in appearance, so it is difficult to accurately tell them apart. This is the case with the *Rastrelliger* mackerel. Two of the mackerel species, *R. brachysoma* and *R. kanagurta*, are very difficult to classify by morphological analysis because of morphological ambiguity. Fortunately, these days, DNA barcoding, which is a relatively new technique for fish authentication, is also being used. A number of researchers observed that the mitochondrial cytochrome oxidase I (*COI*) gene DNA barcode was a powerful tool for species identification [5-6]. In this research, we successfully identified mackerel species using morphological characteristics together with DNA barcoding. Moreover, we showed that the application of molecular techniques together with morphological methods improved the accuracy and precision of mackerel species identification. Our work will be useful for bloodstock selection in mackerel breeding in the future.

2 Materials and methods

The animal study protocol used was reviewed and approved by The Animal Care and Use Committee of King Mongkut's Institute of Technology Ladkrabang, Thailand (ACUC-KMITL-RES/2021/009). Thirty fish samples were obtained from the port in Pathiu, Chumphon Province, Thailand. The collected samples were preserved in an ice box and transported to the laboratory for analysis. The fish species were first identified by morphometric analysis following the methods of [1] and [7] (Fig. 1).



TL = Total length, FL = Fork length, SL = Standard length,
HL = Head length, BH = Body height, and OD = Operculum depth

Fig. 1. Morphological measurement of *Rastrelliger* sp.

The caudal fins of the mackerel samples were subsequently isolated and subjected to DNA extraction. Each DNA sample was visualized in 1% agarose gel stained with SYBR Safe DNA Gel Stain. The concentration and purity were evaluated using a NanoDrop™ Lite Spectrophotometer by reading the absorbances at 260 and 260/280 nm, respectively. The DNA was amplified in the conserved region of the *COI* gene, which consisted of 700 bp, using the barcoding primers [5]:

Forward 5'-TCAACCAACCACAAAGACATTGGCAC-3',

Reverse 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'.

The PCR reaction was performed in a total volume of 50 µl mixture consisting of 100 ng of DNA, 1X Standard Taq Reaction buffer, 0.5 mM MgCl₂, 0.1 mM dNTPs mixture, 10 pmol of forward and reverse specific primers, and 1.25 U Taq DNA polymerase. Amplification conditions were 95°C for 3 min followed by 30 cycles at 95°C for 30 s, 54°C for 30 s, 72°C for 60 s, and then a final extension of 72°C for 10 min. The amplified products were analyzed with 1.5% agarose gel stained with SYBR Safe DNA Gel Stain. All PCR products were sent for DNA sequencing at Macrogen, Inc. (Seoul, Republic of Korea). Subsequently, the DNA barcoding of each sample was compared to the reference sequences in GenBank using the BLAST (<https://blast.ncbi.nlm.nih.gov>) and BOLD systems (<https://www.boldsystems.org>) for species identification. Multiple alignment was performed using BioEdit version 7.0.5.3 [8]. Subsequently, a phylogenetic tree was constructed using the neighbor-joining method by MEGA version 10.2.6 [9], where the *COI* sequences of accession nos. MK423229.1, EF607536.1, and KJ502081.1 from the GenBank database were used as reference sequences for *R. brachysoma*, *R. kanagurta*, and *Selaroides leptolepis*, respectively. *Selaroides leptolepis* was selected as an outgroup.

3 Results and discussion

The species of samples were initially identified by morphological analysis based on the methods of [1] and [7], and the results are shown in Table 1 (Morphological identification). These results indicated that there were 16 *R. brachysoma* and 14 *R. kanagurta*. The physical characteristics of *R. brachysoma* and *R. kanagurta* are demonstrated in Fig. 2. *R. brachysoma* had a very deep body and the body depth was in the range of 37.14-46.45 mm. The head length was equal to or less than the body depth and it was in the range of 42.20-45.59 mm. Moreover, the fork length was 3.6 to 4.1 times the operculum depth (Forked length/Operculum depth, Table 1). Its intestine was very long and 3.4 to 5.7 times the fork length (Intestine length/ Forked length, Table 1). The caudal and dorsal fins were yellowish with a black rim. The pectoral fin was pale yellow. The pelvic and anal fins were a silver-grey color. *R. kanagurta*, however, had a slimmer face. Its head length was longer than its body depth. The head length was in the range of 36.17-43.74 mm, while the body depth was in the range of 29.65-38.62 mm. The body of *R. kanagurta* was slim and the fork length was 4.5 to 5.1 times the operculum depth (Forked length/Operculum depth, Table 1). The intestine length was 0.8-1.9 times the fork length (Intestine length/ Forked length, Table 1). Black spots were found along its body near the dorsal fin. The pectoral, caudal, and dorsal fins were yellowish-grey. The report from [1] revealed that *R. brachysoma* had a relative fork length to body depth of 3.7-4.3 and intestine length to fork length ratio of 3.2-3.6. On the contrary, *R. kanagurta* showed a relative fork length to body depth of 4.3-5.2 and intestine length to fork length ratio of 1.4-1.8.

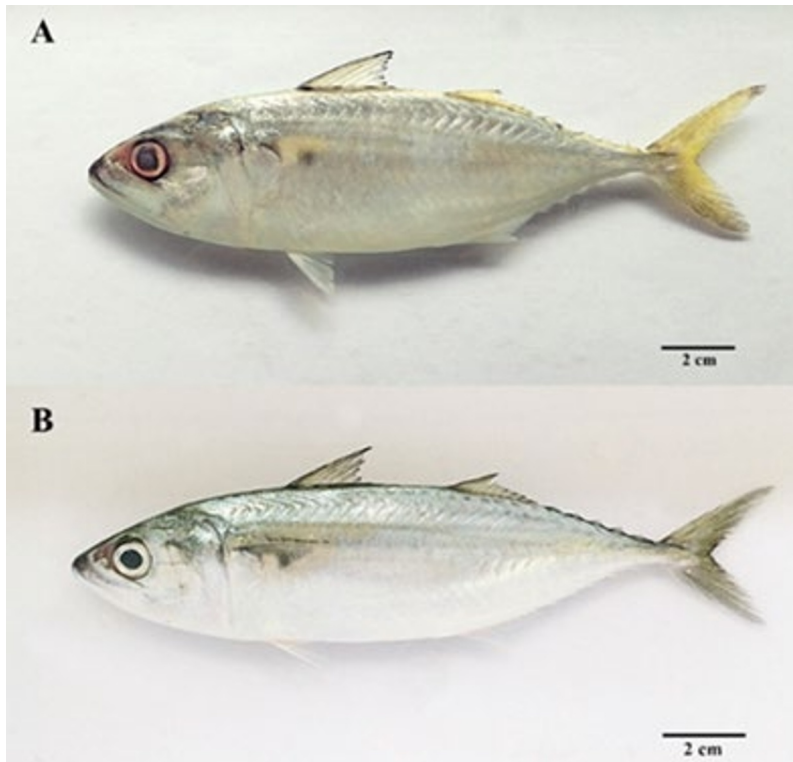


Fig. 2. Morphological characteristics of A) Short mackerel, *R. brachysoma*, and B) Indian mackerel, *R. kanagurta*.

All fish samples were subsequently identified to the species by molecular analysis. A DNA barcode of the *COI* gene was used for the species identification. The total length of the *COI* fragment gene was 621 bp for both *R. brachysoma* and *R. kanagurta*. The *COI* sequences of all samples were compared with the reference sequences in GenBank and BOLD systems. The species was confirmed when each *COI* sequence had a similarity value to the relevant reference sequence in both databases in the range of 99-100%. The identified species by DNA barcoding are shown in Table 1 (DNA barcoding identification). A phylogenetic tree was constructed to illustrate the phylogenetic position of *R. brachysoma* and *R. kanagurta* (Fig 3). The samples of *R. brachysoma* and *R. kanagurta* were prominently defined into two major clades, which correlated with the species identification based on genetic analysis shown in Table 1.

The results shown in Table 1 and Fig. 3 revealed that for the 30 samples, 22 samples were distinguished with correspondence of morphological and genetic analysis (73%). However, there were 8 samples for which morphological identification was inconsistent with genetic assignment (27%). Seven samples (R.b.-5,-14,-16,-17,-18,-27,-30) that had been identified as *R. brachysoma* by morphological analysis were re-assigned as *R. kanagurta* after genetic analysis, and one sample (R.k.-13), which had been assigned as *R. kanagurta* on the basis of morphological analysis was re-assigned as *R. brachysoma* after genetic analysis. Species misidentification of broodstock may induce unintended interspecific hybridization, which could have a negative repercussion on embryonic development and viability. Conversely, such interspecific hybridization may give rise to progeny with phenotypic advantages over their parental strains, thereby threatening the persistence of native species [10]. Furthermore, species misidentification can adversely impact the

conservation of species at risk of extinction, and lead to inaccurate population assessments, which in turn undermine the implementation of appropriate resource-management and conservation targets [11].

Accurate species identification is critical in biodiversity and fisheries management. Identification of fish species has traditionally been based on external morphological characteristics. However, in the case of fish types that have similar characteristics and various developmental stages, identification by morphological characteristic can be difficult [12]. DNA barcoding is a useful method in overcoming such fish species identification problems as it is applicable to individual fish at all stages of life and to all carcass pieces [13-14]. Our research and conclusions agree with the findings of previous researchers [13, 14-15] who used DNA barcoding to confirm fish species identification based on morphometric characteristics. It is clear then that DNA barcodes can be used to confirm the accuracy of identification of fish species based on morphological appearance.

Table 1. Species identification by morphological method and DNA barcoding.

No	Sample code	Morphological identification			DNA barcoding identification				
		FL/OD	IL/FL	Identified species by morphology	BOLD ID	Identity (%)	GENBANK ID	Identity (%)	Identified species by DNA barcoding
1	R.b.-1	4.0	4.7	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>
2	R.b.-2	3.8	4.3	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.68	<i>R. brachysoma</i>
3	R.b.-4	3.9	5.3	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.68	<i>R. brachysoma</i>
4	R.b.-5	4.1	4.1	<i>R. brachysoma</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
5	R.b.-13	4.0	4.0	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.68	<i>R. brachysoma</i>
6	R.b.-14	4.0	4.0	<i>R. brachysoma</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
7	R.b.-16	3.7	3.7	<i>R. brachysoma</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
8	R.b.-17	3.6	3.6	<i>R. brachysoma</i>	<i>R. kanagurta</i>	99.84	<i>R. kanagurta</i>	99.51	<i>R. kanagurta</i>
9	R.b.-18	3.7	3.7	<i>R. brachysoma</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
10	R.b.-20	3.7	3.7	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.52	<i>R. brachysoma</i>
11	R.b.-21	3.7	3.7	<i>R. brachysoma</i>	<i>R. brachysoma</i>	99.84	<i>R. brachysoma</i>	99.51	<i>R. brachysoma</i>
12	R.b.-24	3.7	3.7	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.52	<i>R. brachysoma</i>
13	R.b.-25	3.7	3.7	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.68	<i>R. brachysoma</i>
14	R.b.-27	4.0	4.0	<i>R. brachysoma</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
15	R.b.-29	3.8	3.8	<i>R. brachysoma</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.51	<i>R. brachysoma</i>
16	R.b.-30	3.7	3.7	<i>R. brachysoma</i>	<i>R. kanagurta</i>	99.84	<i>R. kanagurta</i>	99.35	<i>R. kanagurta</i>
17	R.k.-2	4.9	1.6	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
18	R.k.-3	5.0	1.1	<i>R. kanagurta</i>	<i>R. kanagurta</i>	99.84	<i>R. kanagurta</i>	99.51	<i>R. kanagurta</i>
19	R.k.-4	4.7	1.7	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.19	<i>R. kanagurta</i>
20	R.k.-5	4.8	1.9	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
21	R.k.-7	4.5	1.8	<i>R. kanagurta</i>	<i>R. kanagurta</i>	99.83	<i>R. kanagurta</i>	99.51	<i>R. kanagurta</i>
22	R.k.-9	5.0	1.6	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.67	<i>R. kanagurta</i>
23	R.k.-12	4.7	1.6	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.03	<i>R. kanagurta</i>
24	R.k.-13	4.6	0.8	<i>R. kanagurta</i>	<i>R. brachysoma</i>	100	<i>R. brachysoma</i>	99.51	<i>R. brachysoma</i>
25	R.k.-14	4.9	1.9	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.67	<i>R. kanagurta</i>
26	R.k.-15	4.9	1.9	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.51	<i>R. kanagurta</i>
27	R.k.-16	4.8	1.6	<i>R. kanagurta</i>	<i>R. kanagurta</i>	99.83	<i>R. kanagurta</i>	99.51	<i>R. kanagurta</i>
28	R.k.-17	5.1	1.9	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
29	R.k.-20	4.8	1.9	<i>R. kanagurta</i>	<i>R. kanagurta</i>	100	<i>R. kanagurta</i>	99.68	<i>R. kanagurta</i>
30	R.k.-21	4.9	1.5	<i>R. kanagurta</i>	<i>R. kanagurta</i>	99.30	<i>R. kanagurta</i>	99.35	<i>R. kanagurta</i>

Note: FL, Forked length; IL, Intestine length; OD, Operculum depth

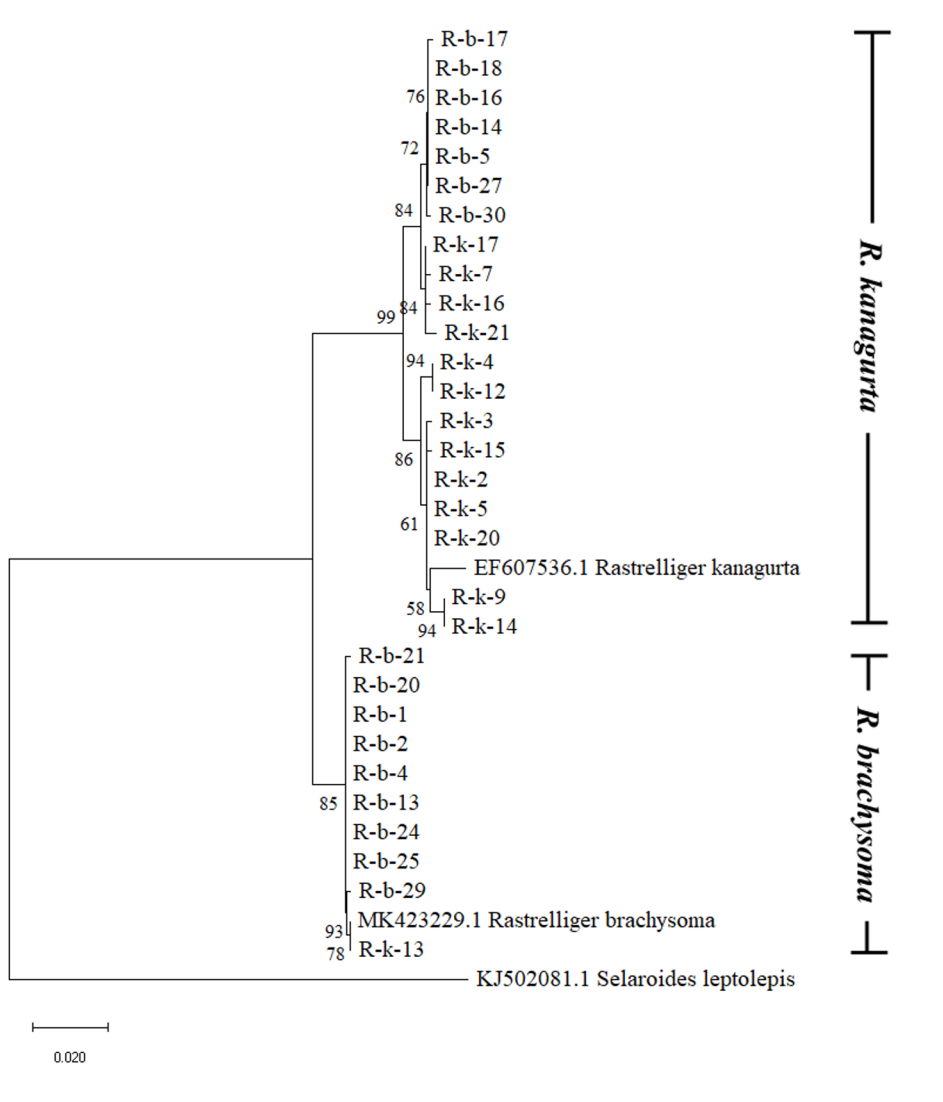


Fig. 3. Phylogenetic tree created based on the *COI* sequences. Numbers above branches represent percentages of bootstrap values.

4 Conclusions

This research was carried out to identify the 2 species in *Rastrelliger* sp.: *R. brachysoma* and *R. kanagurta*, by morphometric and genetic (DNA barcoding) analysis. Accurate species identification was indicated by both methods. The results revealed that the 22 samples were clearly identified by both methods (73%). However, there were 8 samples for which morphological identification was inconsistent with genetic analysis (27%). Therefore, combined morphometric-genetic analysis is a powerful method for distinguishing the *R. brachysoma* and *R. kanagurta* species. In this study, we successfully identified mackerel samples from the port in Chumphon Province, Thailand, to the species level. The research should be of benefit in future selective breeding programs.

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