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Validation of Microsatellite Markers for *Lutjanus russellii* Species Complex

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Abstract

Lutjanus russellii serves as an important food fish resource for artisanal coral reef fisheries throughout the Indo-Pacific region. The species has been recognized for 2 color morphs between the western Pacific and Indian Oceans. Based on the high degree of nucleotide differences in *Cytochrome oxidase I*, these 2 morphs were recently suggested to be 2 different species; *L. russellii* and *L. indicus*, respectively. Here, a cross-species amplification using 6 microsatellite markers, previously developed for *L. carponotatus*, and validity tests of 19 microsatellite markers, previously developed for *L. russelliii*n Zhanjiang Harbor, South China Sea, were performed on *L. russellii* and *L. indicus* sampled from the Gulf of Thailand and the Andaman Sea. The study showed a successful cross-species amplification in 4 *L. carponotatus* microsatellite loci, whereas 5 of the 19 loci previously developed for *L. russellii* from Zhanjiang Harboren countered amplification failure. Of the 18 loci with amplification success, 16 were found to be polymorphic (*Na* = 4 - 27; *He* = 0.195 - 0.965), each of which contained private alleles in each species complex ranging from 0 -13, and R_{ST} ranging from –0.003 - 0.543.This study evaluated microsatellite markers useful for the investigation of population genetic structures, reef recruitment patterns, and species hybridization of the 2 sister species around the Indo-Pacific Oceans boundary. The results also suggested the existence of local specific polymorphisms, as well as genetic distinctiveness, among these species complex.

Keywords: Cross-species amplification, genetic diversity, genetic distinctiveness

Introduction

Lutjanus russellii (Bleeker 1849) or the Moses perch, is a reef associated fish, distributed in the Indo-West Pacific region [1]. The reproductively mature adults inhabit inshore and offshore reefs and rocky areas, while juveniles are found in brackish mangrove estuaries and the lower reaches of freshwater streams [2-4]. The diet composition of *L. russellii* consists of 73.4 % decapod, 15.3 % fish, 9.5 % benthiccrustacean, and 2.1 % mollusk [5]. *Lutjanus russellii* serves as an important food fish resource for artisanal coral reef fisheries throughout its distribution range. It is commonly marketed fresh or live in Hong Kong and China [6], and is one of the most important commercially caught fish in Australia, with total landings in 1998 to 1999 exceeding 62 tons [7].

Lutjanus russellii was found to show allopatric color morphs between the Indian Ocean and the western Pacific Ocean [1]. The remarked morphological difference between the 2 morphs is the series of

narrow yellow-to-brown stripes on the body, which is present in the Indian Ocean morph, but absent in the western Pacific Ocean morph. In addition, the black spot on the posterior back below the anterior rays of the soft dorsal fin is present in a slightly different position. In the Indian Ocean morph, the spot is mainly well above the lateral line, whereas in the western Pacific Ocean morph, the spot is approximately bisected by the lateral line. Based on the high degree of nucleotide differences in the *Cytochrome oxidase I* (*COI*) gene, these 2 color morphs were recently suggested to be 2 different species: *L. indicus* and *L. russellii*, respectively [8]. *Lutjanus russellii* was believed to be distributed in the western Pacific, from the eastern Thai-Malay Peninsula, east to Fiji, north to Japan, and south to Australia, and *L. indicus* was believed to be distributed in the Red sea and eastern Africa, west to the western Thai-Malay Peninsula [8]. Herein, we refer to *L. russellii* and *L. indicus* as the *L. russellii* species complex.

Thirty-four polymorphic microsatellite loci from *L. russellii* were isolated and characterized using samples from Zhanjiang Harbor, South China Sea [9], 7 of which were found to be useful for studying the larval recruitment pattern in its congeneric species, *L. carponotatus*, in the Keppel Islands, Australia [10]. Despite the microsatellite markers that have been developed, little is known about the *L. russellii* population's genetic structure and its recruitment patterns. Additionally, the possible speciation mechanisms separating *L. russellii* and *L. indicus* have not yet been investigated, especially for samples that are distributed in the Indo-West Pacific boundaries.

To assist the examination of these important issues, this study performed cross-species amplification and validity tests for a total of 25 microsatellite loci in both *L. indicus* and *L. russellii*, using samples collected from the Gulf of Thailand and the Andaman Sea. The evaluation aimed to discover microsatellite markers which are specific for the species complex and contain moderate to high levels of polymorphisms. The results of this study provided useful nuclear genetic markers for evolutionary and ecological studies of the *L. russellii* species complex, studies that are important for aiding the management of sustainable coral reef fisheries.

Materials and methods

Sample collection and species identification

Lutjanus russellii and *L. indicus* samples were collected from coastal zones of the Gulf of Thailand and the Andaman Sea. The fin samples were preserved in 1 % Sarcosyl-Urea solution (1 % Sarcosyl, 8M Urea, 20 mM Sodium Phosphate, 1 mM EDTA). Genomic DNA was extracted using an E.Z.N.A Tissue DNA Kit (OMEGA bio-tek, USA). The *COI* DNA barcoding was employed to confirm species identification. The sequence references for *L. russellii* included KC130830, KC130841, and KC130842, and those for *L. indicus* included EU148539 and EU148540 [8].

The *COI* gene was amplified using the primers FishF2_t1 5'-TGT AAA ACG ACG GCC AGT CGA CTA ATC ATA AAG ATA TCG GCA C-3' and FR1d_t1 5'-CAG GAA ACA GCT ATG ACA CCT CAG GGT GTC CGA ARA AYC ARA A-3' [11]. The PCR reactions were carried out in a total volume of 25 μL, containing 50 mM Tris pH 9.2, 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 2 % DMSO, 0.1 % Tween-20, 0.2 mM $dNTP_3$, 10 pmol of each forward and reverse primer, 1U Go-Taq Flexi DNA Polymerase (Promega), and 100 ng gDNA template. The thermal cycle profile consisted of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were purified using a Gel/DNA fragment extraction kit (Geneaid) and sent to Macrogen Inc. in Korea for sequencing. Each sequence was checked for accuracy with its sequence chromatogram, using BioEdit version 7.0.9.0 [12]. All unambiguous sequences were submitted to Genbank (the given accession numbers being KF830873, KF830882-5, KF830892, KF830894, KF830916-17, KF830919-20, KF830922-23, KF830925-6, KF830929, KF830931-33, KF830937-9, KF830941-3, KF830945-9, KF830951-3, KF830955-6, and KF841451-74). The sequences were aligned using Clustal X version 1.83 [13], giving the multiple alignment 569 bp in length. Levels of nucleotide polymorphisms and divergence were estimated using DNAsp version 5.1 [14]. jModelTest version 0.1.1 [15] was used to estimate the best fitted substitution model, based on the corrected Akaike information criterion (AICc) [16]. The recommended model was HKY. Maximum likelihood analyses were performed using PhyML [17]. The maximum likelihood trees were initiated by BioNJ trees and

bootstrapped with 1,000 replicates. Bayesian inference phylogenies were created using MrBayes version 3.1.2 [18]. The analyses ran for 750,000 generations, at which time the 2 parallel chains had reached convergence (average standard deviation of split frequencies < 0.01), with a sampling frequency of 10. The first 25 % of the sampled trees were discarded as burn-in. Based on the phylogenetic analyses, 29 samples were identified as *L. russellii* and 30 samples were identified as *L. indicus*.

Microsatellite amplification and analyses

A total of 25 microsatellite loci were evaluated for their validities. Six loci were previously developed for their congeneric species, *L. carponotatus* [10]: Lca053, Lca059, Lca103, Lca109, Lca130, and Lca167, and the other 19 loci investigated were previously developed for *L. russellii* using the samples from Zhanjiang Harbor, South China Sea [9]: Lru001, Lru002, Lru003, Lru004, Lru010, Lru011, Lru012, Lru014, Lru019, Lru021, Lru024, Lru025, Lru029, Lru030, Lru034, Lru036, Lru041, Lru042, and Lru043. Each microsatellite locus was PCR amplified in 20 µl volume of 50 mM Tris pH 9.2, 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 2 % DMSO, 0.1 % Tween-20, 0.2 mM $dNTP_3$, 10 pmol of each forward and reverse primer, 1U Go-Taq Flexi DNA Polymerase (Promega), and 100 ng gDNA template. The annealing temperature was optimized for each locus using a temperature gradient from 50 - 70 °C. The thermal cycle began with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, the annealing temperature for 20 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products from the successful amplifications were mixed with the denaturing buffer (10 mM NaOH, 0.05 % bromophenol blue, 20 mM EDTA in formamide) in 1:2 ratios, and run on 6 % denaturing 7 M urea, 0.5X Tris-Borate-EDTA polyacrylamide gels at a constant 50 watt at 50 °C for 1.5 - 4 h (depending on the locus). The gel was silver-stained following the protocol described by Creste and colleagues [19]. The 50 bp molecular weight DNA ladder (New England BioLabs) was included after every 15 lanes of samples. The length and the number of base pairs of each allele were estimated based on the results from GeneTools (Syngene) and on the information on the allele sizes and types of the repeat motif, reported by Guo and colleagues [9] and Harrison and colleagues [10].

Twenty nine *L. russellii* and 30 *L. indicus* samples were screened for polymorphisms on the successfully amplified microsatellite loci. The number of alleles, allelic richness, the number of private alleles, and the observed and expected heterozygosity for each microsatellite locus were estimated using FSTAT [20] and GDA [21]. The inbreeding coefficients within populations (*Fis*) were used to test for deviations from the Hardy-Weinberg equilibrium (HWE) by the Markov chain method, under 10,000 dememorization steps, 20 batches, and 5,000 iterations per batch, using GENEPOP [22]. R_{ST} values were estimated and tested for significance by the Markov chain method, under 10,000 dememorization steps, 100 batches, and 5,000 iterations per batch, using GENEPOP [22].The probabilities of null alleles, allelic drop out, and stutter bands were tested using MICRO-CHECKER [23]. Probability values from multiple tests were corrected based on the Bonferroni correction [24].

Results and discussion

DNA *barcoding of L. indicus* and *L. russellii*

The phylogenetic tree based on 569 bp of the *COI* sequences revealed 2 monophyletic clusters of *L. russellii* and *L. indicus* (**Figure 1**). The 2 species showed approximately 4.57 % nucleotide divergence (*Da*) consistent to what was reported by Allen and colleagues [8]. The sequence alignment of the *L. russellii* samples (n = 32) contained 22 substitutions with 0.544 % nucleotide diversity (π), whereas that of the *L. indicus* samples (n = 32) contained only 3 substitutions with 0.109 % nucleotide diversity (π). Interestingly, within the *L. russellii* lineage, a separation of 2 monophyletic groups with 2.57 % nucleotide divergence (*Da*) was supported by the 100 % maximum likelihood bootstrap and the Bayes posterior probability of 1. This suggested that species classification within the *L. russellii* lineage should be further investigated [8].

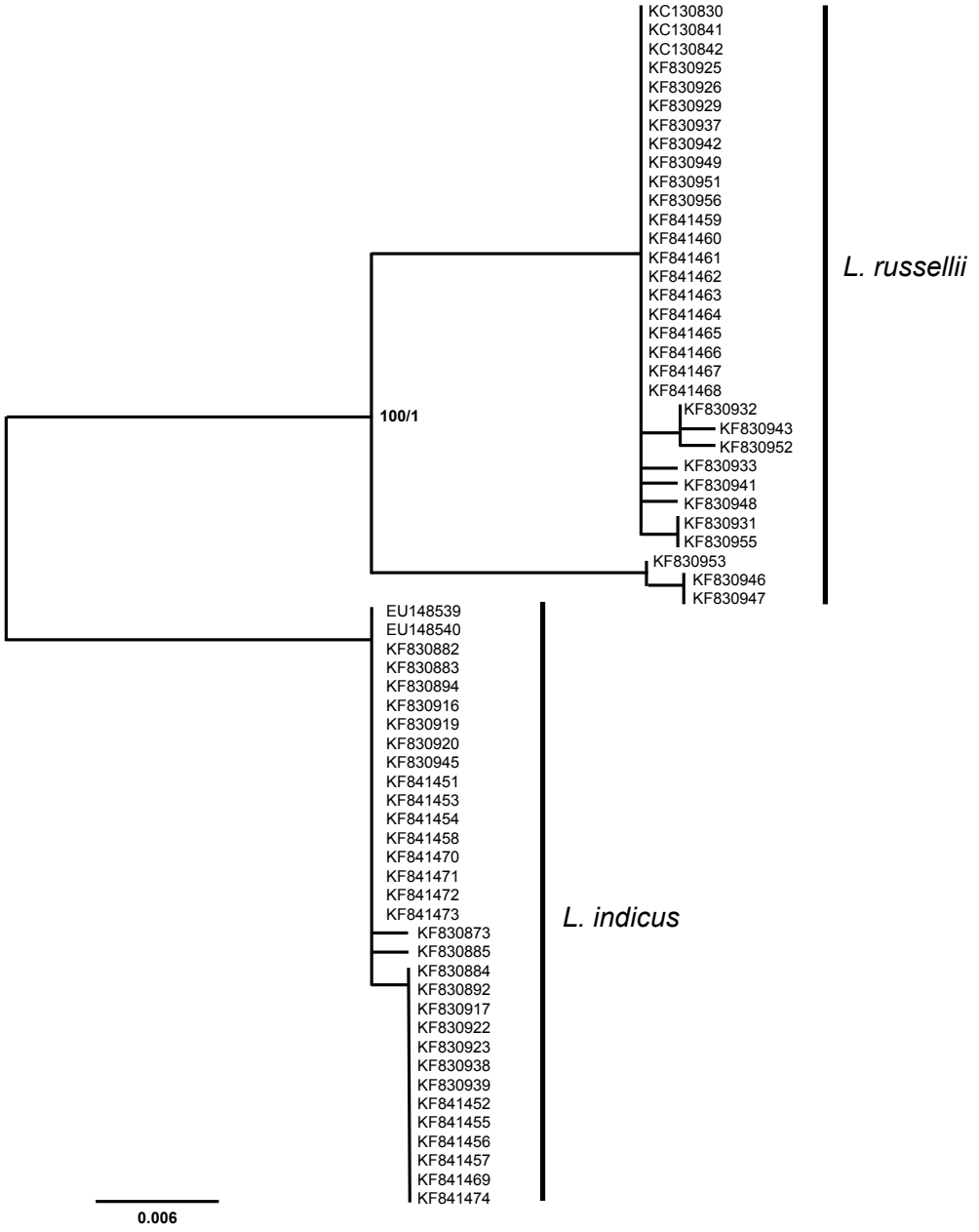

Figure 1 The phylogenetic tree of the *L. russellii* species complex based on *COI* using the HKY model for nucleotide substitution. Support values for maximum likelihood bootstrap ≥ 80 % and Bayes posterior probabilities ≥ 0.70 are shown. The scale bar represents the number of nucleotide substitutions per site. The accession numbers KC130830 and KC130841-42 are sequence references of *L. russellii*, and EU148539-40 are those of *L. indicus*, which were retrieved from GenBank. The other accession numbers are the sequences submitted to GenBank from this study. The accession numbers KF830925-6, KF830929, KF830931-33, KF830937, KF830941-3, KF830946-9, KF830951-3, KF830955-6, and KF841459-68 were grouped with *L. russellii*, whereas the accession numbers KF830873, KF830882-5, KF830892, KF830894, KF830916-7, KF830919-20, KF830922-23, KF830938-9, KF830945, KF841451-8, and KF841469-74 were grouped with *L. indicus*.

Amplification success

Of the 25 loci investigated, 18 were successfully amplified and gave specific amplicons in both *L. russellii* and *L. indicus* (**Table 1**). Four of the 6 *L. carponotatus* microsatellite loci; namely, Lca053, Lca130, Lca167, and Lca103, were successfully cross-species amplified. However, Lca103 was found to be monomorphic in both *L. russellii* and *L. indicus*, leaving only 3 valid *L. carponotatus* microsatellite loci (50 % of tested loci) for the *L. russellii* species complex distributed in the Gulf of Thailand and the Andaman Sea. In general, the success of cross-species amplifications was found to show a negative relationship with phylogenetic distance, by which the success is higher in congeneric than non-congeneric species [25-28]. Additionally, the potential for successful amplification was found highest in species with long generation times, in mixed or out crossing breeding systems, and where genome size in the target species is small compared to the source [29]. The levels of cross-species amplification success varied dramatically across taxa. Among fishes, the percentage of polymorphic microsatellite loci, amplified using primers from different species within the same genus, ranged from 19 - 100 %, with an average of 60 % [30-39]. Based on this estimation, the performance of *L. carponotatus* microsatellite loci for the *L. russellii* species complex was somewhat consistent with the average success.

Interestingly, not all of the microsatellite loci previously developed for *L. russellii* in Zhanjiang Harbor, South China Sea can be amplified in *L. russellii* in the Gulf of Thailand and the Andaman Sea. Of 19 *L. russellii* loci investigated, 5 showed amplification failures (Lru004, Lru012, Lru021, Lru036, and Lru042). In addition, one loci that was successfully amplified (Lru041) was found to be monomorphic (**Table 1**), leaving 13 polymorphic loci (68 % of tested loci) that are valid for the *L. russellii* species complex in the Gulf of Thailand and the Andaman Sea. This level of amplification success is surprisingly low considering that the loci tested were isolated from the same species. Nevertheless, examples of high variation in performance of the microsatellite loci between multiple geographic populations of the same species were reported in snails (genus *Littorina*) [27] and newts (genus *Lissotriton*) [40]. This finding suggests a high number of local specific mutations in the microsatellite flanking regions among *L. russellii* populations/species complex.

Valid microsatellite loci

Of the 25 loci tested, 16 were found to be polymorphic (*Na* = 4 - 27; *He* = 0.195 - 0.965, **Table 1**). All microsatellite loci tested showed the same levels of polymorphisms in both *L. russellii* and *L. indicus*. The loci that are highly polymorphic in *L. russellii* are also highly polymorphic in *L. indicus.* Additionally, all 16 polymorphic loci showed similar levels of gene diversities to that reported by Guo and colleagues [9] and Harrison and colleagues [10]. The tests for deviations from the Hardy-Weinberg equilibrium, however, did not always show consistent results across the *L. russellii* species complex (**Table 1**). Two loci (Lca130 and Lru014) were found to show significant heterozygote deficiencies only in *L. russellii*, and a different locus (Lru030) was found to show a significant heterozygote deficiency only in *L. indicus*. Nevertheless, none of these 3 loci were significantly deviated from the Hardy-Weinberg equilibrium in the Zhanjiang Harbor samples [9]. On the other hand, Lru019 was found to significantly deviate from the Hardy-Weinberg equilibrium in the Zhanjiang Harbor samples, while it appeared in equilibrium in both *L. russellii* and *L. indicus* in the Gulf of Thailand and the Andaman Sea. Among the valid 16 loci, Lru029 is the only locus that was significantly deviated from the Hardy-Weinberg equilibrium in *L. russellii* and *L. indicus*, as well as the Zhanjiang Harbor samples [9]. The possibilities of null alleles, allelic dropout, and stutter bands at each polymorphic locus were tested (**Table 2**). The results showed that none of the loci studied was significantly affected from allelic dropout or stutter band problems, but null alleles were suggested at a few loci. Lru014 was found to show significant possibilities of null alleles in both *L. russellii* and *L. indicus*. Moreover, null alleles were also suggested at Lca053, Lca130, and Lru029 in *L. russellii*, and at Lru034 and Lru030 in *L. indicus*. The inconsistent results of the Hardy-Weinberg equilibrium and null allele tests across the *L. russellii* species complex could be explained by sampling errors and/or evolutionary history, as well as the population genetic structure of the *L. russellii* species complex. The non-random mating between groups would lead to an accumulation of different null alleles in each group. Both *L. russellii* and *L. indicus* were found to contain private alleles at almost all of the 16 polymorphic loci studied (*Pa* = 0 - 13; **Table 2**). The R_{ST}

statistics at each locus ranged from –0.003 - 0.543. Significant R_{ST} was found in 6 loci namely; Lca130, Lru002, Lru019, Lru029, Lru030, and Lru034. The valid microsatellite markers evaluated herein will be useful nuclear genetic markers for the investigation of population genetic structure, recruitment patterns, and species hybridization of the 2 species complex, especially for those distributed along the Pacific-Indian Ocean boundary.

Table 1 The descriptive statistics of 25 microsatellite markers tested for in *L. russellii* and *L. indicus* samples. *Na* number of alleles; *He* expected heterozygosity; *Ho* observed heterozygosity; *Fis* inbreeding coefficient; $^{***}P_{corrected} \leq 0.001$; $^{**}P_{corrected} \leq 0.01$; $^{*}P_{corrected} \leq 0.05$; ^{ns} not significant; NA not applicable; - no data due to amplification failure.

	Locus	Other species/population[a,b]				*L. russellii*				*L. indicus*			
		Na	***He***	***Ho***	***Fis***	***Na***	***He***	***Ho***	***Fis***	***Na***	***He***	***Ho***	***Fis***
Cross-species amplification	Lca053[a]	36	0.920	0.935	-0.010 ^{ns}	21	0.938	0.793	0.156 ^{ns}	19	0.951	0.933	0.019 ^{ns}
	Lca130[a]	25	0.906	0.833	0.028 ^{ns}	16	0.924	0.552	0.407***	25	0.951	0.933	0.019 ^{ns}
	Lca167[a]	29	0.931	0.932	-0.001 ^{ns}	23	0.957	0.965	-0.009 ^{ns}	26	0.965	0.967	-0.002 ^{ns}
	Lca103[a]	40	0.950	0.949	0.000 ^{ns}	1	0.000	0.000	NA	1	0.000	0.000	NA
	Lca059[a]	19	0.705	0.712	-0.010 ^{ns}	-	-	-	-	-	-	-	-
	Lca109[a]	27	0.924	0.922	0.008 ^{ns}	-	-	-	-	-	-	-	-
Validity test	Lru001[b]	14	0.950	0.930	0.021 ^{ns}	20	0.941	0.852	0.096 ^{ns}	19	0.939	0.967	-0.030 ^{ns}
	Lru002[b]	10	0.900	0.890	0.011 ^{ns}	13	0.907	0.899	0.020 ^{ns}	11	0.829	0.867	-0.046 ^{ns}
	Lru003[b]	13	0.955	0.930	0.021 ^{ns}	23	0.955	0.899	0.071 ^{ns}	23	0.956	0.963	-0.008 ^{ns}
	Lru010[b]	14	1.000	0.940	0.060 ^{ns}	25	0.923	0.929	0.036 ^{ns}	24	0.958	0.896	0.065 ^{ns}
	Lru011[b]	14	1.000	0.930	0.070 ^{ns}	22	0.936	0.897	0.043 ^{ns}	20	0.945	1.000	-0.059 ^{ns}
	Lru014[b]	16	1.000	0.950	0.050 ^{ns}	20	0.939	0.708	0.250***	27	0.948	0.767	0.194 ^{ns}
	Lru025[b]	10	0.790	0.840	-0.050 ^{ns}	14	0.904	0.828	0.086 ^{ns}	14	0.838	0.821	0.020 ^{ns}
	Lru030[b]	13	1.000	0.910	0.090 ^{ns}	19	0.942	0.913	0.031 ^{ns}	21	0.950	0.615	0.036***
	Lru043[b]	10	0.850	0.880	-0.031 ^{ns}	19	0.931	0.960	-0.031 ^{ns}	18	0.947	0.867	0.087 ^{ns}
	Lru019[b]	6	1.000	0.640	0.360 ***	8	0.844	0.759	0.103 ^{ns}	11	0.860	0.900	-0.048 ^{ns}
	Lru024[b]	5	1.000	0.680	0.320 ^{ns}	5	0.740	0.586	0.211 ^{ns}	5	0.694	0.600	0.135 ^{ns}
	Lru029[b]	4	1.000	0.550	0.450***	4	0.195	0.069	0.651***	4	0.721	0.567	0.217***
	Lru034[b]	4	0.400	0.570	-0.425 ^{ns}	5	0.632	0.483	0.239 ^{ns}	5	0.699	0.533	0.240 ^{ns}
	Lru041[b]	11	1.000	0.820	0.180 ^{ns}	1	0.000	0.000	NA	1	0.000	0.000	NA
	Lru004[b]	5	0.700	0.740	-0.057 ^{ns}	-	-	-	-	-	-	-	-
	Lru012[b]	12	0.900	0.920	-0.022 ^{ns}	-	-	-	-	-	-	-	-
	Lru021[b]	3	0.920	0.670	0.272 ^{ns}	-	-	-	-	-	-	-	-
	Lru036[b]	9	0.750	0.850	-0.333 ^{ns}	-	-	-	-	-	-	-	-
	Lru042[b]	5	0.700	0.630	0.100 ^{ns}	-	-	-	-	-	-	-	-

[a]Data from 1,154 *L. carponotatus* samples from the Keppel Islands, Australia [10]
[b]Data from 20 *L. russellii* samples from Zhanjiang Harbor, South China Sea [9]

Table 2 The MICROCHECKER results, number of private alleles (*Pa*), R_{ST} values, and genic differentiation test at each polymorphic locus. $^{***}P_{corrected} \leq 0.001$; $^{**}P_{corrected} \leq 0.01$; $^{*}P_{corrected} \leq 0.05$; ns not significant.

	L. russellii				*L. indicus*				R_{ST} (P-value)
Locus	**Null alleles**	**Allelic dropout**	**Stutter bands**	***Pa***	**Null alleles**	**Allelic dropout**	**Stutter bands**	***Pa***	
Lca053	Yes	No	No	3	No	No	No	1	-0.023 (0.102) ^{ns}
Lca130	Yes	No	No	3	No	No	No	12	-0.016 (<0.001) ***
Lca167	No	No	No	4	No	No	No	7	-0.018 (0.386) ^{ns}
Lru001	No	No	No	5	No	No	No	4	-0.018 (0.255) ^{ns}
Lru002	No	No	No	4	No	No	No	2	0.146 (<0.001) ***
Lru003	No	No	No	4	No	No	No	4	0.012 (0.267) ^{ns}
Lru010	No	No	No	7	No	No	No	6	0.154 (0.406) ^{ns}
Lru011	No	No	No	5	No	No	No	3	-0.003 (0.566) ^{ns}
Lru014	Yes	No	No	5	Yes	No	No	12	0.082 (0.126) ^{ns}
Lru019	No	No	No	0	No	No	No	3	-0.011 (<0.001) ***
Lru024	No	No	No	0	No	No	No	0	-0.016 (0.180) ^{ns}
Lru025	No	No	No	5	No	No	No	5	0.037 (0.033) ^{ns}
Lru029	Yes	No	No	0	No	No	No	0	0.358 (<0.001) ***
Lru034	No	No	No	0	Yes	No	No	0	-0.002 (0.001) *
Lru030	No	No	No	11	Yes	No	No	13	0.543 (<0.001) ***
Lru043	No	No	No	4	No	No	No	3	0.177 (0.024) ^{ns}

Conclusions

The validity tests of 25 microsatellite markers for the *Lutjanus russellii* species complex revealed 16 polymorphic loci, 13 of which showed moderate to high levels of gene diversity. These included 3 loci which were previously developed from *L. carponotatus* (Lca053, Lca130, and Lca167) and 10 loci previously developed from Zhanjiang Harbor *L. russellii* (Lru001, Lru002, Lru003, Lru010, Lru011, Lru014, Lru019, Lru025, Lru030, and Lru043). It should be noted that while 50 % of the *L. carponotatus* loci showed a successful cross-species amplification in the *L. russellii* species complex in the Gulf of Thailand and the Andaman Sea, as high as 30 % of the loci previously developed for *L. russellii* from Zhanjiang Harbor encountered amplification failure. Few loci gave inconsistent results for the Hardy-Weinberg equilibrium and null allele tests among the *L. russellii* species complex. These results emphasize the importance of validating microsatellite markers for samples from different geographic regions. Additionally, the study suggests the existence of local specific polymorphisms and genetic distinctiveness within this species complex which require further investigation.

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