Original article

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Nuclear DNA content variation within four species of Asian catfish of family Pangasidae and their two interspecific hybrids by flow cytometry

Variasi konten DNA inti empat spesies ikan patin famili Pangasidae dan dua hibridanya menggunakan *flow cytometry*

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ABSTRACT

Nuclear DNA content (NDC) of species or population is believed to have been formed naturally by many mechanisms, e.g. chromosomal mutation, insertion and deletion, transposable element, and duplication. Additionally, hybridizations and species' phylogenetic relationship may also contribute to the NDC diversity. This study was aimed to investigate the NDC profile in four species of Asian catfishes of the family Pangasidae including *Pangasianodon hypophthalmus*, *Pangasius djambal*, *Pangasius nasutus*, and *Pangasius nieuwenhuisii*, hybrid *P. hypophthalmus* and *P. djambal* (hybrid HD), and hybrid *P. hypophthalmus* and *P. nasutus* (hybrid HN). NDC measurement was performed in an Attune acoustic flow cytometer (ABI) using a DAPI staining and chicken (*Gallus domesticus*) RBC was used as a size reference. The results showed that the NDC mean of *P. hypophthalmus*, *P. djambal*, *P. nasususutus*, and *P. nieuwenhuisii*, were within the range of NDCs in the other catfish families. The NDC of *P. hypophthalmus* was the lowest and was not different from that of hybrid HN ($P>0.05$), but it was different ($P<0.05$) from those of the rests. The NDC of *P. djambal*, *P. nasutus* and *P. nieuwenhuisii* were similar but they were different from that of the hybrid HN. The NDC of both hybrids were significantly different ($P<0.05$). While the NDC of hybrid HD was closer to that of male parent, the NDC of hybrid HW was closer to that of female parent. The phylogeny of Pangasiid catfish species constructed using NDC values in this study was independent of their phylogenetic relationship based on cytoplasmic and nuclear markers.

Keywords: Flow cytometry, nuclear DNA content, *P. hypophthalmus*, *P. djambal*, *P. nasutus*, *P. nieuwenhuisii*, interspecific hybrid.

ABSTRAK

Konten DNA inti (KDI) suatu spesies atau populasi terbentuk melalui berbagai mekanisme seperti mutasi kromosom, insersi dan delesi, transposable element, dan duplikasi. Selain itu, hibridisasi dan posisi spesies dalam filogeni juga berperan memengaruhi keragaman KDI. Penelitian in bertujuan menginvestigasi profil KDI pada empat spesies ikan patin dari famili Pangasidae yang meliputi *Pangasianodon hypophthalmus*, *Pangasius djambal*, *Pangasius nasususutus*, dan *Pangasius nieuwenhuisii*, hibrida *P. hypophthalmus* dan *P. djambal* (hibrida HD), dan hibrida *P. hypophthalmus* and *P. nasutus* (hibrida HN). Pengukuran KDI dilakukan menggunakan *Attune Acoustics Flow cyometer* (ABI), dengan DAPI dan sel darah merah ayam, *Gallus domesticus*, sebagai kontrol standar. Hasil penelitian menunjukkan rerata KDI *P. hypophthalmus*, *P. djambal*, *P. nasususutus*, *P. nieuwenhuisii*, berada dalam kisaran KDI famili *catfish* lainnya. KDI terendah terdapat pada *P. hypophthalmus* yang tidak berbeda ($P>0.05$) dengan KDI hibrida HN namun berbeda ($P<0.05$) dengan spesies/grup lainnya. KDI *P. djambal*, *P. nasutus*, dan *P. nieuwenhuisii* tidak berbeda satu dengan yang lain namun berbeda dengan KDI hibrida HN. Konten DNA inti pada hibrida HD berbeda nyata dengan hibrida HN. Konten DNA inti hibrida HD lebih mendekati KDI tetua jantan, sedangkan KDI hibrida HN lebih mendekati tetua betina. Filogeni spesies catfish yang dibangun menggunakan nilai KDI dalam studi ini tidak mengikuti pola kekerabatan sesuai hubungan filogenetik berdasarkan marka genetik sitoplasmik dan inti.

Kata kunci: *Flow cytometry*, konten DNA inti, *P. hypophthalmus*, *P. djambal*, *P. nasutus*, *P. nieuwenhuisii*, hibrida interspesifik.

INTRODUCTION

Nuclear DNA content (NDC) has been used to state the amount of DNA contained, traditionally expressed in picogram, in haploid genome of organismal cells. It has been used interchangeably with other terms such as cellular DNA content (Jianxun *et al.,* 1991), genome size and C-value (Doležel & Greilhuber, 2010; Salvador-Soler *et al.*, 2016). The nature of NDC within a species is influenced by a variety of forces such as chromosomal mutation, insertion and deletion, transposable element, and duplication (Gregory, 2005). In addition to these, it is also influenced by hybridization, as it may induce rapid changes in gain or loss DNA within the genome (Agudo *et al.,* 2019). In addition to being an effect of these various causative forces, the NDC may serve as a causative force that links or affects organismal phenotypes, through its influence on cell size and cell division rate. Subsequently, the cell size and cell division rate, independently or in an interaction, may affect organismal phenotypes such as developmental rate, metabolic rate, organ complexity and body size (Gregory, 2005).

Interrelationships of the NDC as a causative agent as well as an effect have been explored a lot in plant and animal taxa. In the late 1970s, flow cytometry has become a principal of genome size research (Rana & Jain, 2019). A study by Gruner *et al*. (2010) found that the genome size could be a good predictor of root meristem growth rate (RMGR), in which a negative exponential relationship exist between genome size and RMGR. A study conducted by Vallès *et al.* (2012) uncovered that genome size may be used to develop systematic data in genera of Anthemideae and Cardueae (Asteraceae). A phylogenetic signal of the NDC has also been shown in liverworts, a flowerless, spore-producing plants (Bainard *et al.,* 2013). Being one of most diverse animal taxa, a significant report of NDC studies in fish have also been documented (Gregory, 2005). Several NDC studies in fishes have also tried to uncover interrelationships of NDC as both a causative and as an effect. For instance, in a study with forty two species of Chinese freshwater fishes, it was uncovered that there is a close correlation between nuclear chromosome number or ploidy and DNA content in fish. These observations suggest that the more specialized species, the less nuclear DNA content the fishes own (Jianxun *et al.,* 1991). Zhu *et al.* (2012), who explored the NDC and its possible correlates in eight species of commercially important fishes in China, found that genome size was correlated with erythrocyte nucleus size. Studies of NDC in catfish families have also been documented. The online database of genome size (Gregory, 2019), documented 47 records consisting of 11 families. Of these records, however, no Pangasiid catfish were included.

Pangasiid catfish have been one of major food fishes produced by aquaculture subsector in Indonesia. In 2017 its production figure reached 320,000 tons and became one among the ten species contributing most to the aquaculture production (KKP, 2018). In addition to *Pangasianodon hypophthalmus,* which was introduced from Thailand in 1972, several species of Pangasiid catfishes have been documented in Indonesia, some of which were *P. djambal*, *P. nasutus,* and *P. nieuwenhuisii* (Gustiano *et al.,* 2018, Tahapari *et al.,* 2011). While *P. djambal* has been proposed as a new candidate for aquaculture, the aquaculture prospect of *P. nasutus* have also been started to explore (Iswanto & Tahapari, 2014, Tahapari *et al.,* 2011). Various genetic approaches to improve aquaculture production have been carried out. These mainly included selective breeding and interspecific hybridization. The selective breeding program in *Pangasianodon hypophthalmus,* has successfully produced an improved growth of this strain more than 30%. The interspecific hybridization between female *P. hypophthalmus* and male *P. jambal* has produced a white flesh hybrid called Pasupati that is preferred by consumers (Tahapari *et al.,* 2016). Likewise, the hybrids of female *P. hypophthalmus* and male *P. nasutus* have produced a white flesh hybrid (Darmawan & Tahapari, 2017, Hassan *et al.,* 2011, Iswanto & Tahapari, 2014). This study was aimed at exploring the NDC of four species of Asian catfish, including *P. hypophthalmus, P. djambal, P. nasutus, and P. nieuwenhuisii,* and their two interspecific hybrid combinations, which were male *P. hypophthalmus* × female *P. djambal* and male *P. hypophthalmus* × female *P. nasutus.* An emphasis was also put on the nature of NDC diversity in relation to their phylogenetic relationship.

MATERIALS AND METHODS

Fish samples

Four species of pangasiid catfish, namely *Pangasianodon hypophtalmus*, (sample size, N=10) *Pangasius djambal* (N=10), *P. nasutus* (N=10), *P. nieuwenhuisii* (N=2) and the hybrid

of female *P. hypophtalmus* and male *P. djambal* (hybrid HD, N= 9), and hybrid of female *P. hypophtalmus* and male *P. nasutus* (hybrid HN, N=8*)*, were sampled. The age and size range of individuals belonging to the respective groups were seven months, 0.50 ± 2.03 cm for *P. hypophthalmus*, eleven months and 32.56 ± 2.36 cm for *P. djambal*, ten years and 65–570 cm for *P. nieuwenhuisii*, seven years and 45–50 cm for *P. nasutus*, and nine months and 31.76 ± 1.51cm for the hybrids. The samples of *P. hypophthalmus and P. djambal were* the second and first generation, respectively, while those of *P. nasutus and P. neuwenhusii* were the original stock collected from nature. All samples represent the live collection of the Research Institute for Fish Breeding, a government owned research institute located at Sukamandi, Subang Regency, West Java, Indonesia.

Laboratory analyses

Protocols for determination of NDC, in general consist of three major steps, namely sampling of red blood cell (RBC), preparation of staining solution, and measurement in the instrument. The samples of RBC were taken from both fish sample and a chicken, *Gallus domesticus*, which was used as size standard. The use of chicken RBC as a genome size standard for measurement of the unknown samples in this study was mainly caused by its advantageous features. Its size has been known, the nuclei suspension is homogenous, and it is relatively tolerant to long term storage at low temperature. These characteristics have made it a popular size standard in many studies of genome size (Mendonça *et al.,* 2010). One ml of RBC representing both groups were drawn using a pretreated 3 ml syringe and was processed according our laboratory protocol standard, which was modified from (Zhu *et al.,* 2012). The cell staining used was of 4-,6-diamidino-2-phenylindole (DAPI), a fluorescent stain that has a greater photo stability and strongly binds to adenine-thymine rich region of dsDNA. Preparation of the DAPI staining solution followed the manual described by manufacturer (*Thermo Fisher Scientific*). Measurement of sample and size standard was carried out using an attune acoustic focusing cytometer (Applied Bio system) following a series of optimization to produce clear flow cytometry peak profiles that allow unambiguous scoring.

Data analyses

The data were statistically analyzed to evaluate whether there were differences in the NDC means among different species, between hybrids, and between species and hybrids. Due to the nature of the data, which were low in number, unequal in sample size, and were not normally distributed, a nonparametric Kruskall-Wallis test was implemented (Marusteri & Bacarea, 2010). Post hoc tests of Conover-Inman for all pair wise comparisons were performed when initial Krukall-Wallis test resulted in a statistically significant difference. The analyses were performed in Systat 13. To evaluate whether variation in NDC among Pangasiid species are associated with their phylogenetic relationships, a comparative phylogenetic analysis which focused on the topology of dendrograms was carried out. A dendrogram of Pangasiid catfish constructed using NDC values was compared to those constructed using commonly used markers for phylogeny construction, namely mitochondrial cytochrome oxidase I (mtCOI) and allozyme markers. The NDC-based dendrogram was constructed using agglomerative hierarchical clustering, by applying Euclidean distance and agglomeration method of Unweighted pair-group average. The analysis was implemented in XLstat (Addinsoft 2019), an addin software belongs to excel. To construct the mtCOI-based dendrogram, mtCOI sequences of *P. hypophthalmus*, (accession number EU752151.7) *P. djambal* (accession number KP036427.1) and *P. nasutus* (accession number KT0010451) were retrieved from the National Center of Biotechnology Information (NCBI) website and analyzed. No mtCOI sequences of *P. nuewenhuisii* were available that it was not included in the analyses and in the construction of mtCOI-based dendrogram. The dendrogram was built by applying neighbor joining method implemented using Bioedit version 7 (Hall *et al.,* 2011). The topology of nuclear genome dendrogram was redrawn and simplified from allozyme-based phylogeny provided by Pouyaud *et al.* (1998)

RESULTS AND DISCUSSION

Histogram profiles of flow cytometric peaks

Two distinctive histogram flow cytometric peaks, representing unknown sample and chicken RBC as size standard are observed within each of six groups, namely *P. hypophthalmus, P. djambal,*

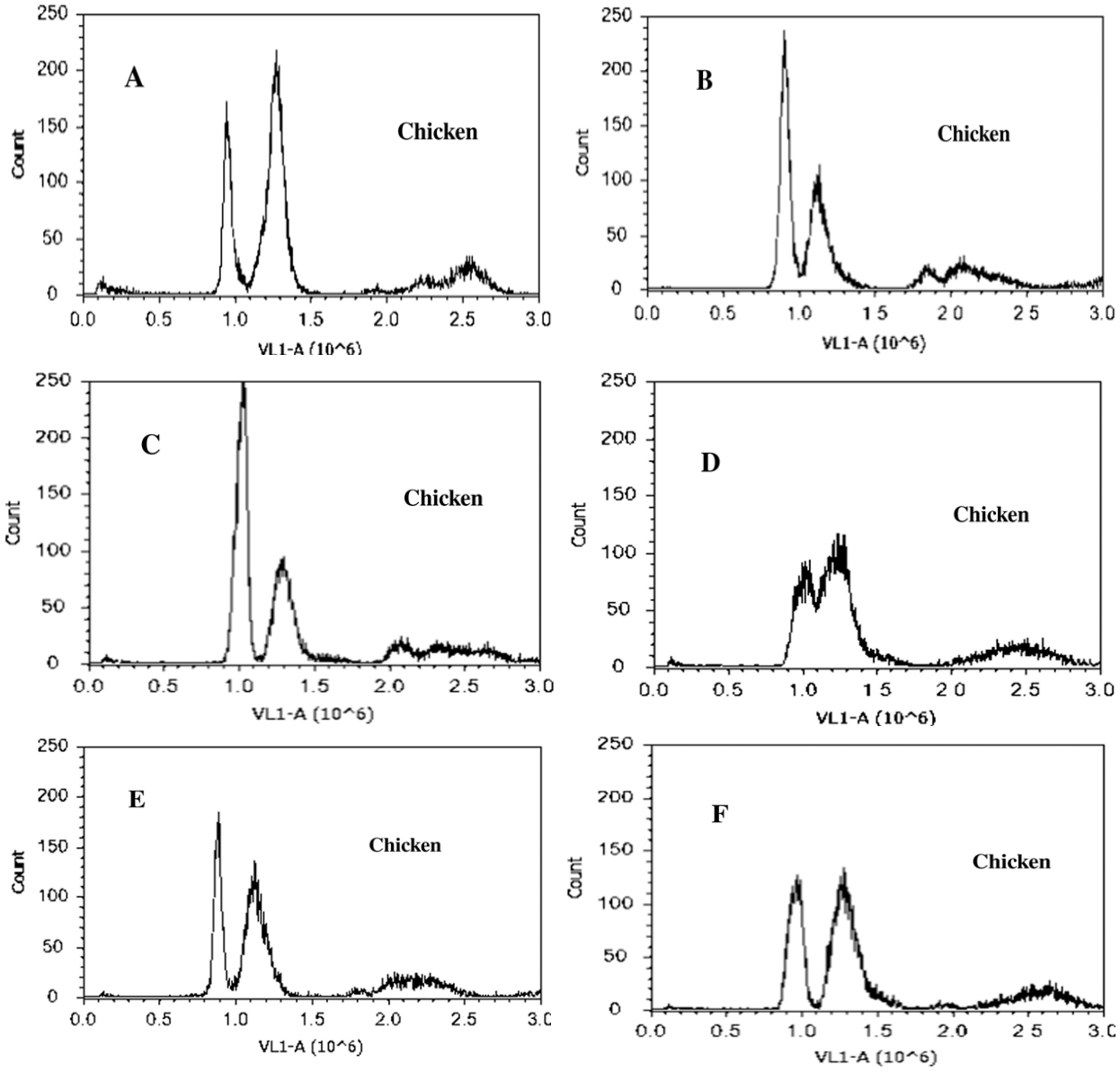

Figure1. Flow cytometric peaks of sample (left) and chicken RBC (right) as size standard in *P. hypophthalmus* (A), *P. djambal* (B), *P. nasutus*, (C) *P. nieuwenhuisii* (D), hybrid HD (E), and hybrid HN (F).

P. nasutus, P. nieuwenhuisii, hybrid HD and hybrid HN (Figure 1). Both peaks are clearly distinctive that scoring can be made without ambiguity. The figures suggest that from technical perspective, the adopted laboratory protocol of genome size analysis, including RBC and staining solution preparations, loading concentration, parameter setting of the instruments and the use of chicken RBC as size standard for measurement of NDC within these groups were appropriate.

Comparative analyses of NDC among groups of Pangasiid catfishes.

The NDC of six groups of Indonesian Pangasiid catfish, consisting of four species and two hybrids are presented in Table 1. It shows that they range from 0.960 pg in *P. hypophthalmus* *to* 1.074 pg in *P. nieuwenhuisii.* In average, the NDC value within these four species was around 1.000 pg. There were only subtle differences in NDC value among species of Pangasiid catfish. However, a statistically significant difference was found in a particular set of pairwise comparisons. Specifically, the NDC of *P. hypophthalmus,* besides being the lowest it was also significantly lower than *P. djambal P. nasutus* and *P. nieuwenhuisii* ($P<0.05$), but it was not significantly different from that of *P. nasutus.* The profile of NDC expressed in megabase show a similar pattern, since the NDC value in megabase is a linier transformation from those expressed in picogram. Another feature characterized the NDC value of Pangasiid catfish was the relatively low

Table 1. Nuclear DNA content, expressed in picogram (pg) and megabase (Mb), in genera Pangasiid of species *P. hypophthalmus, P. djambal, P. nasutus, P. nieuwenhuisii,* hybrid HD, and hybrid HN.

Species/Group	n	Genome size		CV (%)
		pg	Mb	
P. hypophthalmus	10	0.960 ± 0.025[a]	938.600 ± 24.859[a]	2.649
P. djambal	10	1.017 ± 0.050[b]	994.687 ± 49.919[b]	5.019
P. nasutus	10	1.000 ± 0.041[b]	977.535 ± 40.173[b]	4.110
P. nieuwenhuisii	2	1.074 ± 0.023[b]	1050.636 ± 22.598[b]	2.151
Hybrid HD	9	1.005 ± 0.036[b]	982.897 ± 35.050[b]	3.566
Hybrid HN	8	0.956 ± 0.009[a]	934.819 ± 8.708[a]	0.932

Note: CV (coefficient of variation), HD (hybrid of *P. hypophthalmus x P. djambal*) and HN (hybrid of *P. hypophthalmus x P. nieuwenhuisii*).

Table 2. A comparative profile of nuclear DNA content (NDC) values of Pangasiid catfish (bold printed) relative to other catfish families compiled from animal genome database (Gregory, 2019).

Family	NDC range (pg)		Divergence	
	min	max	(%)	folds
Ariidae	2.25	2.47	9.78	1.10
Bagridae	0.92	1.10	19.57	1.20
Clariidae	0.92	1.20	30.43	1.30
Doradidae	1.60	1.60	0.00	1.00
Ictaluridae	0.59	3.00	408.47	5.08
Loricariidae	0.89	2.10	135.96	2.36
Malapteruidae	1.00	1.00	0.00	1.00
Mochokidae	1.10	1.20	9.09	1.09
Pangasidae	**0.96**	**1.07**	**11.46**	**1.11**
Plotosidae	1.75	1.75	0.00	1.00
Schilbeidae	0.98	0.98	0.00	1.00
Siluridae	0.85	1.44	69.41	1.69

level of among-individual variation. As can be seen in Table 1, the coefficient of variation within each species ranged from 2.151% to 5.019%.

In the context of other catfish families, the NDC of Pangasidae produced from the present study was within the range of previously published results (Table 2). Within the scope of catfish families, five folds of NDC values were reported, being the lowest (0.59 pg) and the highest (3.00 pg) were found in the family Ictaluridae. The NDC in Pangasiid catfishes is comparable to other families, except for Doradidae, Plotosidae and Ariidae, which show much higher ranges (Table 2).

The NDC of the hybrids relative to their parental lines

In this study, two interspecific hybrid combinations that were explored their NDC presented two different patterns. The first pattern (Figure 2A) shows that the NDC hybrid was higher and significantly different (P<0.05) from that of the parental female, but it was similar to and not significantly different (P>0.05) from that of the parental male. This occurred in the hybrid HD, in which their NDC value (1.005 pg) was higher than that of the *P. hypophthalmus* (0.960 pg) as the female parent but lower than that of the *P. djambal* (1.017 pg) as their male parent. While the NDC value of hybrid HD was in between of its parental lines, the figure is not precisely intermediate. It was closely laid to the male parent. Different from the first pattern, the second pattern (Figure 2B) shows that the NDC of the hybrid, namely Hybrid HN (0.956 pg) was similar to that of the female parent (*P. hypophthalmus*, NDC= 0.960 pg) but it was lower than and significantly different (P<0.05) from that of the male parent (*P. nasutus,* NDC=1.000 pg).

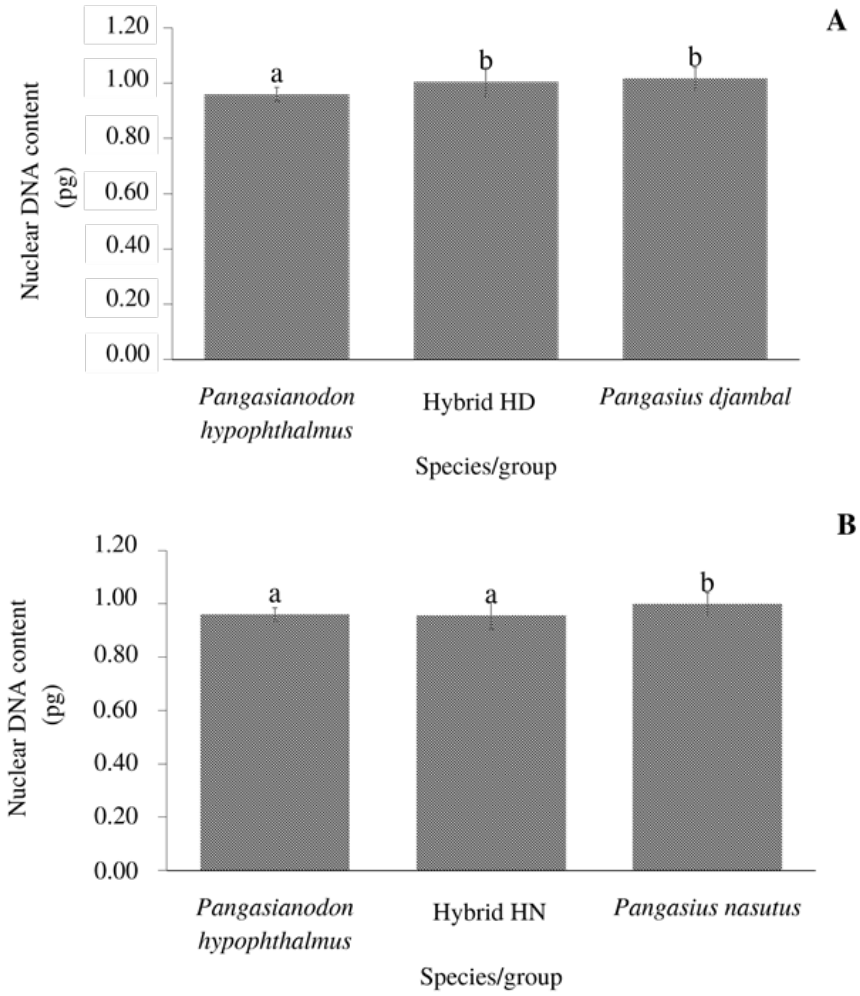

Figure 2. Pattern of nuclear DNA content (NDC) in picogram of two Pangasiid hybrid combinations. Hybrid between female *P. hypophthalmus* and male *P. djambal*, Hybrid HD (A) and female *P. hypophthalmus* and male *P. nasutus*, Hybrid HN (B).

Relatedness of Pangasiid catfish based on the NDC and *mtCOI*, and allozyme markers.

Dendrogram of Pangasiid catfish constructed based on the values of NDC, *mtCOI*, and allozyme markers are presented in Figure 3. Based on the NDC dendrogram (Figure 3A), the closest distance was between *P. hypophthalmus* and *P. nasutus*, the largest distance was between *P. nieuwenhuisii* against the others, while *P.djambal* was laid in between. Different from this pattern, in the dendrograms based on mtCOI (Figure 3B) and based on allozyme (Figure 3C), the closest distance was between *P. nasutus* and *P. djambal*. The largest distance, at least as shown by the allozyme-based dendrogram, was between *P hypohpthalmus* and the others. Looking at the NDC-based and allozyme-based dendrograms, both of which have the same number of taxa, no congruent patterns of topology were observed. In brief, it is apparent that the NDC values in catfishes, in particular with species explored in this study are independent of their previously uncovered relatedness as shown by both cytoplasmic (mtCOI) and nuclear (allozyme) markers.

DISCUSSION

To date, the NDC of 2295 fish species has been documented on genomsize.com, an online resource freely accessible (Gregory, 2019). This number, however, is too small compared to the huge number of fish species, which has been reported at more than 33,000 species. The study of nuclear DNA content in Pangasiid catfishes, to the best of our knowledge, is the first. No other similar results have been reported. Hence, our data contributed and fill the gap of information on NDC or genome size in catfish families. Further, several findings of the present study

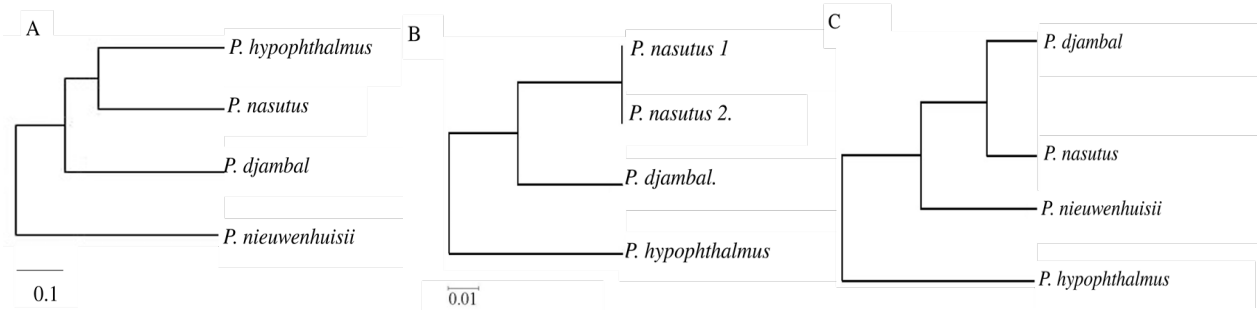

Figure 3. Dendrograms showing the topology of relatedness of Pangasiid catfishes constructed using values of NDC generated from the present study (A), using sequences of mtCOI retrieved from the NCBI (B), and using extracted and simplified information based on allozyme markers phylogeny (C), provided by Pouyaud *et al*. (1998). No distance measures are included in the redrawn picture (C) as it emphasizes more on topology.

are of interest to discuss. These included the profile of intraspecific diversity and interspecific divergence, the NDC profile of the hybrids, and the NDC profile of Pangasiid by taking into account their phylogenetic relationships. Finally, the discussion will be concluded with possible implications these study may have on practical aquaculture.

Intraspecific NDC diversity observed in this study (0.90–5.00%) was low, and was in accordance with the theoretically expected. A study with Family Ictaluridae, Genera *Ictalurus* found an intraspecific diversity of 1.28% (Tiersch *et al.,* 1990). With the exception of salmonids and cyprinids which showed a more dynamics NDC due to chromosomal changes, the major source of intraspecific diversity in other teleost fishes is expected to be related to the sex chromosome (Gregory, 2019). Referring to a study with avian taxa, Mendonça *et al*. (2010) showed that the Z chromosome shows 200% more DNA content than does the W chromosome. This characteristic has allowed the NDC in chicken, *Gallus domesticus*, to be used as a tool to discriminate males from females. The same principle may hold for fishes in which sex- determination system is controlled by sex chromosome. In the female homogametic system of fish, the sex chromosome of female (XX) is assumed to have higher DNA content than that of the male (XY) leading to differentiation in NDC between males and females. On the contrary, in the male homogametic system (ZZ) of fish, the male is thought to have a slightly bigger NDC than that of the female. These principles could be applied to detect the unresolved status of sexually undetermined individuals. While several data, particularly those dealing with plant of Caricaceae species (Gschwend *et al.,* 2013), and birds (Mendonça *et al.,* 2010) have supported the idea, several other works with fish (see e.g. Imron *et al.,* 2019, Zhu *et al.,* 2012) have failed to lend similar support. Whether a similar pattern, in which NDC difference between male and female, would emerge from Pangasiid catfish remains to be explored. In contrast to intraspecific diversity, there are more possible factors inducing the interspecific NDC divergences.

Interspecific NDC divergences may rise as result from chromosome size variation, chromosomal proliferation and ploidization, such as the case with sturgeons (Havelka *et al.,* 2011) and penaied shrimps (Swathi *et al.,* 2018). The pattern of interspecific NDC divergence in Pangasiid catfish seemed to resemble that was observed in other catfish families excluding Siluridae and Loracariidae. Within these families, a significant divergence ranging from 1.7 to 5.0 folds in NDC is observed, while in the remaining families, the degree of divergence was much lower (less than 1.3 folds). With respect to Pangasiid, the highest within-family divergence was around 12%. By looking at these figures, the polyploidization event as a causative factor causing NDC divergence in Pangasiid can be omitted. If polyploidization was the case for the variety of genome size in Pangasiid, the lowest degree of genome size divergence would be at least 50%, which was not supported by the data. This pattern, in which genome sizes were found to be relatively similar within genera, has also been discovered in other studies (see e.g. Stetter & Schmid, 2017). Hence, other inducing mechanisms such as the dynamics and evolution of transposable elements and hybridizations may serve as alternative explanations.

Artificial hybridizations, as exemplified in the present study, have proven to diversify the NDC in Pangasiid. Nevertheless, the patterns were not consistent, being similar to male parent in one

case but resembling female parent in the other combination. The widely accepted theory with regards to genome size of diploid hybrid is that they would be in between those of their parental lines. This was based on a thought that the genome size of a hybrid is simply the combinations of haploid chromosome set derived from their parents. There have been many evidences supporting this theory. A study of interspecific hybrids in plants commercial lines of *Lotus uliginosus* Schkuhr and *Lotus corniculatus* found that genome size of the hybrids had an intermediate value (Castillo *et al.,* 2012). Working with hybrid catfish generated from three genera of family Ictaluridae, Tiersch and Goudie, (1993) found that the genome size of the hybrid was precisely in between of those of the parental lines which suggest that nuclear DNA segregates as a function of a haploid DNA content and it is stable within intergeneric hybrid. A similar pattern was also observed in interspecific hybrid within the genus *Poeciliopsis*, in which genome size of hybrid between *Poecilia monacha* and *P. lucida* was intermediate between those of the progenitors (Dawley *et al.,* 1997). A disagreement profile of NDC Hybrid HN to that theoretically expected may be associated with chromosomal pairing. The diploid (2n) chromosome number of *P. hypophthalmus* was 60 (Sreeputhorn *et al.,* 2017) while that of *P. nasutus* was 2n=58 (Alassan, 2007). This difference in a number of chromosomes within the haploid genome of parental species may induce incomplete chromosomal pairing in the resulting hybrid.

Traditionally, phylogenetic studies have been carried out using molecular genetic markers, such as allozyme, mitochondrial DNA or nuclear DNA. The allozyme is an initial molecular markers widely used in studies of population's genetic (Allendorf 2017), including phylogenetics (see e.g. Pratt *et al.,* 2011, Rao *et al.,* 2017). The mitochondrial genetic markers, in the form of a whole genome or of genes comprising it, such as D-loop genes or cytochrome oxidase genes, have been widely used to capture and to resolve phylogenetic relationships (Allendorf 2017). Several studies (see e.g. Li *et al.,* 2017, Zhu *et al.,* 2017) have proven that mtDNA based phylogenies are powerful in delineating relatedness in various taxa. In terms of nuclear DNA, various nuclear ribosomal genes such as 18S rRNA and 16S rRNA have been widely used in Phylogenetic studies (reviewed by Patwardhan *et al.,* 2014). In contrast to the previously mentioned markers, very few studies that elaborated and compared the phylogenetic patterns resulting from the NDC to those constructed using mitochondrial and allozyme markers have been reported.

Based on mitochondrial marker, *P. djambal* and *P. nasutus* which belonged to genus *Pangasius* formed one clade and were separated from *P. hypophthalmus* which belonged to genus *Pangasianodon.* Based on allozyme markers, a similar profile was shown. *P nieuwenhuisii*, *P. djambal*, and *P. nasutus* which belonged to genus *Pangasius* formed a closely related group and were separated from *P. hypophthalmus* of genus *Pangasianodon.* In brief, the phylogenies constructed from mitochondrial and allozyme markers were congruent in their topology. However, they were incongruent when it was compared to that constructed using the NDC. There are numerous factors that may explain phylogenetic conflict resulting from different markers. These include differences in evolutionary process or history, biological and methodological sources (Davalos *et al.,* 2012). With respect to the NDC phylogeny of Pangasiid generated from the present study, showing a lack of congruency with those constructed using more established phylogenetic markers (mtCOI and allozymes), all the above mentioned explanations are possible. To get a more firm insight on the most plausible explanations, a specific study, involving more number of Pangasiid species that have been recognized (Gustiano *et al.,* 2018), is required.

The NDC information by mean of flow cytometry in fish may have practical implications to aquaculture, the most notable being in the estimation of ploidy levels. For the purposes of improving quality associated with sexual maturation such as higher growth rates, stronger disease resistance, and better organoleptic properties, triploid fish has been an interesting option for aquaculture (Hu *et al.,* 2019). As a result, an accurate technique for determination ploidy status is a need. There are several methods of detecting ploidy levels, one of them is DNA content determination with flow cytometry (Kim *et al.,* 2017). It has been the most effective, rapid, and accurate method to identify the ploidy status in fish (Xavier *et al.,* 2017). In general, the use of NDC determination by flow cytometry to evaluate or to verify ploidy status is similar to that for determination of genome size. The main difference is laid in the size standard being used. In determination of genome size, a size standard of known size (for example a chicken RBC), is used that genome size of unknown sample

can be determined. In determination of ploidy status, a reference sample of known ploidy status, commonly a normal diploid possessing two chromosomes (2n), is used as size standard that ploidy level of a sample of unknown ploidy status, for example a triploid (3n) or a tetraploid (4n) can be determined. This is allowed as samples possessing different ploidy levels will be displayed as different flow cytometric peaks (see e.g. Çakmak *et al.,* 2019) as an example. The degree of peak differences between samples differing in their ploidy which is proportional to the ploidy level allows determination of respective samples. Other possible practical applications of fish NDC for aquaculture can be explored by studying its relationships with traits of economically important. These relationships have been explored quite extensive in plants, for example for traits RMGR (Gruner *et al.,* 2010). In animal groups however, such relationships have not been strongly supported (Gardner *et al.,* 2020). Therefore, more studies on the relationship between the NDC and other traits important to aquaculture need to be examined that beneficial relationships can be identified. Based on the known relationships between NDC and the traits of interest, aquaculture engineering taking benefit of that relationship can be implemented.

CONCLUSION

The NDC of four species of Pangasiid catfishes and their two interspecific hybrids, ranging from 0.956 pg to 1.074 pg, were within the range of previously reported NDC values for other families of catfish. The highest and the lowest NDCs were found in *P. hypophthalmus* and *P. nieuwenhuisii,* respectively. The NDC patterns of both hybrids were different, in that the NDC of hybrid HD was closer to that of the male parent, while the NDC of hybrid HN was closer to that of the female parent. Relatedness of Pangasiid catfishes based on NDC was not congruent with those constructed using mtCOI and allozyme markers.

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