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Research Article

Dojo Loach *Misgurnus anguillicaudatus* (Cantor 1842): An Addition to the Loach Fauna of Pakistan

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Abstract | Some fish were found in June 2013, buried at a depth of 4-5 inches in a sandy loam textured soil, having an average moisture content of 21% in the Botanical Garden of the University of Peshawar. Preliminary investigations suggest that the fish is classified within the genus *Misgurnus*. To confirm and further identification, a total of 77 specimens were collected. A batch of nine specimens was randomly selected. Thirty morphological characters along with meristic counts were recorded and studied against four different norms. Standard I consisting of ratios of various meristic parameters was 100% concurrent with our data, while for standard II, nine parameters were scrutinized and 77.77% of the result was falling in the expected array. Standard III was simply a correlation formula application. The value for "Y" was (-0.3) which rest in the array of Dojo loach (*Misgurnus anguillicaudatus*). The fourth norm based on hematology was also almost exactly alike with diploid *M. anguillicaudatus.* In addition, the morphological appearance, presence of asterisks, microscopic dusky spots on body, low adipose crest, lateral upper bands, presence of lamina circularis, absence of mid-lateral stripe from eye to caudal base and the absence of a narrow stripe from the opercula to caudal fin or at least up to pelvic origin and meristic parameters confirmed the new reported species as diploid *M. anguillicaudatus*.

Novelty Statement | This is the first report of *Misgurnus anguillicaudatus* from Pakistan. The species was directly collected from moist soil rather than water. Current study also reports morphometric and meristic counts along with haematological parameters of *M. anguillicaudatus* from Pakistan.

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Introduction

On June 7, 2013, a significant number of an unidentified fish species were discovered buried approximately 4-5 inches below the surface at the Centre of Plant Biodiversity, University of Peshawar, Azakhel, Nowshera, Pakistan. Specimens were brought to the Fisheries Lab, Department of Zoology (now IZS), University of Peshawar, Pakistan. The amphibious fish was first identified as a Loach on the basis of its morphology and pattern of circumoral barbles. As no taxonomic identification key of Pakistan contained any information about it, hence by matching it

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with the images of loaches on different websites, it was suggested to be a member of genus *Misgurnus*. Another closely related genus; *Paramisgurnus* was also described on websites, and it was not easy to certify just by matching with pictures that the fish belongs to genus *Misgurnus* or not. Still identification up to species level was a serious issue because of the occurrence of ploidy, introgression and extreme variations in Asian weather loaches. The main aim of this study was to identify the newly recorded fish from Pakistan up to species level.

Misgurnus was first described by Cantor from Chusan Island (China) in 1842 (Simon *et al*., 2006). It belongs to family Cobitidae and is regarded as native to China, Hong Kong, Japan, Korea, Russia and Vietnam. The species has also been introduced and naturalized in many other countries including Australia, Italy, US along with Hawaii, Germany and Turkmenistan while currently their population have been introduced in Grand Calumet River, Lake Michigan drainage and Indiana harbor canal (Simon *et al*., 2006). There are many other important species of the genus *Misgurnus* (Lacepede, 1803), of which *M. fossilis* is native to Europe while the remaining *M. mohoity, M. nikolskyi, M. buphoensis, M. tonkinensis, M. anguillicaudatus, M. mizolepis* and *Paramisgurnus dabryanus* are native to East Asia.

All these fish are members of the family Cobitidae and are generally known as loaches, which are benthic and widely distributed across Europe, Asia, and many other parts of the world (Conway *et al*., 2011). The loaches are distributed at various altitudes ranging from 50m below the contour line up to an altitude of 5200m (Slechtova *et al*., 2007). The adaptation of loaches to different environments has led to a diversity of species (Sato *et al*, 2011). The loaches are much diverse and the problem of taxonomy of loaches is still debated and remains unresolved. Among loaches, Chinese loaches hold a very significant position ecologically, economically as well as medicinally and *M. anguillicaudatus* (Cantor, 1842) is one of them (Milton *et al*., 2018).

Misgurnus anguillicaudatus are slender and eel like with mottled body, having five pairs of barbels (Kessel *et al*., 2013). They can tolerate temperatures between 2°C to 30°C (Urquhart, 2013). They are commonly used by anglers as live bait, so escapes may also have contributed to their spread (Allen *et al*., 2002). They live generally in swamplands, lakes, rice fields and streams. They are omnivores with preference towards snails, worms and insect larvae (Simon *et al*., 2006). They prefer a little bit highly oxygenated water respiring through their gills. They are highly adaptive to every sort of environment because of potent respiratory habit as they respire through their skin and posterior straight portion of intestine as well as engulf air to supplement respiratory requirements in oxygen-depleted water by coming above the water surface while excrete respiratory gases through anus (Allen *et al*., 2002).

The invasiveness and potential impact of *M. anguillicaudatus* on ecosystems is not much explored. However noteworthy reduction in macro invertebrates' number and biomass has been observed in their presence. They are allied to eminent nitrate, ammonia and turbidity level (Keller and Lake, 2007). *M. anguillicaudatus* in China is represented by two independently occurring forms; diploid and tetraploid.

Loaches are medicinally significant and are used in the treatment of various skin diseases. Likewise, *M. anguillicaudatus* is very much important in China because of its use in traditional medicines as well as for food purposes. Its culture output has reached up to 204,552 tons in 2010 in main land of China (ncbi.nlm.nih.gov/pmc/articlePMC3577766).

A polysaccharide isolated from mucus of this fish called MAP (*M. anguillicaudatus* polysaccharide) showed anti-proliferative and apoptotic effects on human cancer cell lines (Zhang and Huang, 2005). The polypeptide MAPP (*M. anguillicaudatus* antimicrobial polypeptide) has also exhibited antimicrobial activities against various bacteria (Dong *et al*., 2002). Some other peptides called hepcidin and misgurin have also been identified from the whole body homogenates of *M. anguillicaudatus,* which are used medicinally. In China they have been used in folk medicines for curing hepatitis, carbuncles, osteomyelitis, inflammation and cancers (Qin *et al*., 2002).

Here, we provide new record for *M. anguillicaudatus* species captured during the ichthyological exploration in Nowshera, Pakistan which is not included in the previous compilations of fishes known to occur in the country.

Materials and Methods

The sampling was done from Centre for Plant Biodiversity, Azakhel, Nowshera situated at 34°00.398 North and 71°52.574 East. A total of three collections were made successively: first in July, second in August while the last collection was made in September 2013. The specimens were brought to Fisheries Lab, Department of Zoology (now IZS), University of Peshawar and kept in different aquaria labeled as aquarium I, II and III.

Before conducting hematological studies, the fish were kept in high oxygen concentration and fasted for two days before taking the blood samples. A thin smear was made over sterilized slide using a small drop of blood taken from the caudal peduncle. The slides were then air dried, fixed and stained. The slides were then observed through oil immersion lens of a microscope (Leitz DIALUX 22

EB, Japan). Measurements of different blood cells were taken using stage micrometer and ocular micrometer; mean and standard deviations were calculated for these values. Photographs of the blood cells were captured by using digital microscope camera (Scopetek DCM 300). The specimens were then kept in 100% methanol for morphometric study. Morphometric measurements were taken by using ruler and vernier caliper. The measurement of each body part was taken while scales and other meristic elements were counted. The fin rays of dorsal and anal fin were very small, so they were observed under stereomicroscope (MEIJI No.159393, Japan) after partial removal of skin fold. The specimens were cataloged and kept in the natural history museum, Department of Zoology (IZS), University of Peshawar.

Results and Discussion

Morphometric measurements

In this study four different standards (Shimizu and Takagi, 2010; Fowler, 1924; Nichols, 1925; Koizumi *et al*., 2007; Gao *et al*., 2007) were used for comparison. A total of 30 different morphometric parameters were considered in the study. Table 1 shows all these parameters and concerned values taken on nine specimens.

Standard- I (Shimizu and Takagi, 2010)

The reference data for comparison was taken from Shimizu and Takagi, 2010 who first differentiated the two closely resembling species *P. dabryanus* and *M. anguillicaudatus* by analyzing their mtDNA and then distinguished their morphometrics. To confirm the identification of the fish under consideration 15 parameters were selected and their percentages and ratios were calculated against Standard Length (SL), Head Length (HL) and Caudal Peduncle Length (CPL) (Table 3). All the characters were similar with that of Shimizu and Takagi (2010) except one parameter i.e. the percentage of caudal peduncle depth against caudal peduncle length, which showed a little deviation from the range, but still it was similar with the reference standard when its standard deviation was taken under consideration.

Table 1: Morphometric parameters taken on nine specimens.

Parameter (mm)	Abb	S1	S2	S3	S4	S5	S6	S7	S8	S9
Total Length	TL	96	110.5	116	111	103	123	105	102	131.5
Standard Length	SL	84	95	101	97	86	104	88	86	112
Pre-dorsal Length	PL	47	52	53	53	46	57	50	50	60
Post Dorsal Length	PDL	54	63	62	63	54	67	41	51	69
Pre Pelvic Length	PVL	51	53	55	57	48	51	60	60	64
Head Length	HL	12.7	14.5	16	16	14	16.5	14	13	17
Snout Length	SnL	6	6	6.5	6	6	7	6	5.3	7
Post Orbital Length	POL	6	7.5	9	8	7	9	7	7	10
Eye Diameter	ED	2	2.2	2	2	2	2	2	2	2.2
Length of Caudal Fin	CFL	12	15.5	15	14	17	19	17	16	19.5
Length of Base of Dorsal Fin	DFBL	7	11	9	10	8	10	11	11	9
Length of Caudal Peduncle	CPL	15	20	19	20.5	17	21.5	15	16	22
Depth of Caudal Peduncle	CPD	6.8	10.5	9	11	8.5	7.2	9	9.5	15
Body Depth	BD	10.5	13	12	13	9	16	14	12	16
Pre Pectoral Length	PPL	14	14	15	16.5	14	16	17	17	17
Pre Anal Length	PAL	62	64	65	67	57	68	63	59	77
Pectoral Fin Length	PFL	14	14	15	16.5	14	16	16	17	17
Head Width	HW	7	6.5	7.5	6.5	6	9	7	14	12
Inter Orbital	IO	3	4.5	4	4	3.7	4.5	4	3.5	6.3
Dorsal Fin Height	DFH	14.5	16	17	15	14	18	14	13	17.5
Longest Pectoral Fin ray	LPFr	16	16	16	19	16	18	17	19	19
Pectoral Fin Base Length	PFBL	4.5	4	4	4	3.5	5	4	3	5
Anal Fin Length	AFL	14	15	16	15.5	17	17	15	12	18
Anal Fin Base Length	AFBL	7	9.5	10	9.5	8	10	9	8	11
Maxillary barbel	MAX	7.8	7.5	7.5	6	6.5	8	6.5	6.5	7
Longest Dorsal ray	LDr	11.5	12	12	11	11	14	12	10	12
Longest Anal ray	LAr	10	11	12	11.5	12	12	11	9	10
Head Depth	HD	10.5	11.5	12	11	8	11	14	14	15
Body Width	BW	6	6	8	7	6	8	11	11	16

Table 2: Meristic Counts of the nine specimens.

	S1	S2	S3	S4	S5	S6	S7	S8	S9
Dorsal fin	1r,1/7,I or II,7,I	1r,1/7,I or II,7,I	II,7,I or 1r,I/7,I	1r,1/7,1 or II,7,I	1r,1/7,I or II,7,I	II,7,I or 1r,I/7,I	1r,1/7,I or II,7,I	II,7,I or 1r,I/7,I	1r,1/9
Anal fin	II,6.I or II,6	II,6.I or II,6	II,6.I or II,6	1r,1/6 or 1,7,1	II,6.I or II,6	II,6.I or II,6	II,6,I	II,6.I or 1h,1r/6,I	1r,1/6,1r
L.L scales	155	155	135-145	135-145	135-145	155	145	145-150	146-150

Table 3: Comparison of morphometric counts with Schimizu and Takagi (2010)/

Percentage against SL	Mean ± SD	Shimizu and Takagi, 2010
SL	94.8±9.62*	74.0-93.0*
HL	15.7±0.52*	16.3-17.2*
BD	13.4±1.7*	12.0-17.0*
CPL	19.4±1.4*	16.9-18.9*
CPD	10.1±1.9*	9.3-12.4*
PL	55±1.7*	55-59.6*
DFBN	10.1±1.7*	7.6-10.4*
VL	16.6±1.8*	17.3–19.6*
PAL	68.3±2.9*	71.1–74.2*
Percentage against head length		
SnL	41.8±2.2*	34.1-43.3*
OD	13.8±1.3*	11.9-13.8*
Percentage against caudal peduncle length		
CPD	52.2±9.9*	55.0-73.2*
Ratios		
SL/MBD	7.5±1*	5.9-8.4*
SL/CPD	10.2±2.1*	8.1-10.8*
CPL/CPD	1.9±0.4*	1.4-1.8*

The symbol (*) shows that the observed values are same with Shimizu and Takagi, 2010.

Hence according to this standard the fish has been identified as *M. anguillicaudatus* Table 3. Percentage of standard length (SL), head length (HL) and caudal peduncle length (CPL) against various body parts and its comparison with Schimizu and Takagi (2010).

Standard- II (Fowler, 1924; Nichols, 1925)

In this standard the observed parameters for the fish in the current study was compared with values and ratios mentioned by Fowler (1924) and Nichols (1925) for the identification of *M. anguillicaudatus*. The morphometric ratios calculated were 77.77% coincident with Fowler (1924) and Nichols (1925) for *M. anguillicaudatus* as compared to any other species. The parameters and their results are given in the Table 4.

Meristic counts

The meristic counts of the specimens for dorsal and anal fins were measured, which were similar with that of Fowler (1924). The number and size of the Dorsal and anal fin rays were checked by stereomicroscope (20X), such that the fin's skin was partly removed after cutting the fins from fish body. The Dorsal fin rays were: 1 rudimentary, 1 single, 7 branched, 1 rudimentary or II,7, I and anal were: 1 very small or rudimentary type, 1 was single, 6 were branched and again the last one was rudimentary or simply II, 6, I. Similarly, the lateral line scales up to caudal fin of nine specimens were counted, number of which ranged from 135 or 140-155, which resembled with Fowler (1924) as according to him the number of scales in *M. anguillicaudatus* ranges from 138-170.

Table 4: Comparison of observed parameters with Fowler (1924) and Nichols (1925).

Morphometric ratios	Mean±SD	Fowler (1924)* and Nichols (1925)**
SL/HL	6.4±0.2*	6.4-6.8* ≥6 or ≤6**
HL/SnL	2.4±0.14*	2.6-2.8*
HL/E	7.2±0.73*	5.8-7.2*
HL/MAX	2.1±0.29X	3.7-4*
HL/IO	2.9±0.26X	4.8-5.2*
SL/BD	7.5±1.0*	7.3-9* b/w 7 and 8**
SL/HD	8.17±1.4*	b/w 7 and 8**
Caudal fin length	15.7±1.9*	= to or slightly>HL**
HL	14.4±1.4	= TO Or slightly< CF**

Furthermore, the color of the specimens was dull brown after keeping the specimens in alcohol along with more distinction of color of the upper surface as compared to lower surface.

Standard – III (Koizumi et al., 2007)

Differentiation formula b/w *P. dabryanus* and *M. anguillicaudatus* by Koizumi *et al.* (2007). Koizumi *et al.* (2007) has given a simple formula to differentiate between *M. anguillicaudatus* and *P. dabryanus* in Japan. They first differentiated both the species through DNA sequencing and then made a simple discrimination by using measurements of six body parts i.e. SL, HL, BD, CPD, CPL, LB. The means of CPD/SL%, CPL/SL% and LB/SL% in the *P. dabryanus* were significantly larger than those of the *M. anguillicaudatus*, while the means of HD/SL% and BD/SL% did not remarkably differ between these species. They applied the following formula.

$Y = 0.241\ \text{CPD/SL\%} + 0.721\ \text{LB/SL\%} - 8.140$

(if $Y \leq 0$: Dojo loach, $Y > 0$: Kara-dojo loach)

Table 5: Number of observed dorsal, anal fin rays and scales.

	S1	S2	S3	S4	S5	S6	S7	S8	S9
Dorsal Fin	1r,1/7,I or II,7,I	1r,1/7,I or II,7,I	II,7,I or 1r,I/7,I	1r,1/7,1 or II,7,I	1r,1/7,I or II,7,I	II,7,I or 1r,I/7,I	1r,1/7,I or II,7,I	II,7,I or 1r,I/7,I	1r,1/9
Anal Fin	II,6.I or II,6	II,6.I or II,6	II,6.I or II,6	1r,1/6 or 1,7,1	II,6.I or II,6	II,6.I or II,6	II,6,I	II,6.I or 1h,1r/6,I	1r,1/6,1r
Scales	155	155	135-145	135-145	135-145	155	145	145-150	146-150

Table 6: Simple correlation formula (Koizumi *et al.*, 2007) application on our specimens.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	Mean ± SD
CPD/SL%	8.1	11.1	8.9	11.3	9.9	6.9	10.2	11.0	13.4	10.09 ± 1.9
LB/SL%	9.3	7.9	7.4	6.2	7.5	7.7	7.4	7.5	6.25	7.47 ± 0.9

It provides a precise alternative method for species identification which relies only on three measurements without the need of any other morphological or DNA sequence analysis keeping in view that the specimens were measured after the initial shrinkage by circa of one month by keeping them in absolute (99.5%) ethanol.

The Caudal Peduncle Depth (CPD)/Standard Length (SL %) was calculated. Similarly, Length of the Barbel (LB)/Standard Length (SL %) was also computed, then the values were put in the following formula:

Y = 0.241 CPD/SL% + 0.721 LB/SL% - 8.140
Y = 0.241×10.09+0.721×7.47-8.140
Y = -0.3

The result (-0.3) lies in the range of the Dojo loach (*M. anguillicaudatus*) rather than Kara Dojo loach (*P. dabryanus*) hence it confirms that the fish under consideration is *M. anguillicaudatus*.

Standard – IV (Gao et al., 2007)

Haematology: The standard for the evaluation of blood cell parameters was taken from Gao *et al.* (2007). For further confirmation of the species a group of nine specimens was taken and their blood cells measurements were acquired (in µm). Mean of each blood cell type is given in Table 7.

Table 7: Comparison of the size of blood cell.

	Mean ± SD (µm)	Gao *et al.* (2007)
RBC Nuclear length	5.47±0.39	4.66±0.34µm
RBC Nuclear width	2.98±0.28	3.01±0.11µm
Thrombocytes length	6.42±1.15	6.81±0.87µm
Thrombocytes width	5.16±0.95	5.85±0.86µm
Neutrophils Length	10.77±1.7	12.60±1.46m
Neutrophils width	9.01±1.18	11.29±1.16m

Morphology of blood cells

In the peripheral blood smear of the loach, erythrocytes were the most abundant cells, while leucocytes (thrombocytes, neutrophils and monocytes) were scattered diffusely amid the dense erythrocytes.

Erythrocytes

There were two kinds of erythrocytes found in loaches i.e. mature and immature erythrocytes. The mature erythrocytes were elliptical cells in shape with an oval, central purple stained nucleus and a blue-gray stained cytoplasm. The surface of the cells was nearly smooth. The reticulocytes were somewhat round in shape with fewer number and non-clear cytoplasmic and nuclear membranes. Dividing erythrocytes were also found but they were very scarce in number. The length (mean) of the nucleus of erythrocytes was 5.47±0.39 µm falling in the range of triploid *M. anguillicaudatus* while its width was 2.98±0.28 µm, which was falling in the range of diploid *M. anguillicaudatus*. Both these parameters were deviating least as compared to other blood cells values.

Thrombocytes

The shape of thrombocytes was round/oval and appeared as single cells. They stained dark purple. The number of thrombocytes was highest in first collection, lower in 2nd and lowest in 3rd collection. The length of thrombocytes was 6.42±1.15 µm while its width was 5.16±0.95 µm, which was falling in the range of diploid *M. anguillicaudatus*. Deviation from mean for thrombocytes was more than erythrocytes but less than neutrophils.

Neutrophils

The shape of neutrophils was variable from spherical to ovoid and pear shaped while the shape of its nucleus was ovoid and horseshoe shaped. The position of nucleus was always eccentric. The neutrophils stained light purple while its nucleus stained dark purple. The size of neutrophils was variable and larger than erythrocytes. The mean length of neutrophils was 10.77±1.7 µm and their width was 9.01±1.18 µm, respectively. The size of neutrophils was largest among all the blood cells. The density of neutrophils was higher in freshly excavated fishes while lower in old excavated fishes.

A total of nine fish specimens were taken for the morphometric measurements. They were apparently of different age groups most probably ranging from 1-3 years of age based on the available data i.e. (Maximum length observed in nature is 280mm while common length is 150mm. The age at first maturity in *M. anguillicaudatus* is 1 year for male while 2 years for females (Kessel *et al*., 2013). The mature female has a fuller abdomen than the male of the same size while the male form lamina circularis in the pectoral fin at maturity. Our specimens had a minimum total length of 96mm and maximum Total Length of 131.5mm whilst minimum standard length was 84mm and maximum standard length was 112mm (Table 1), this much discrepancy in total length and standard length was due to the disparity in their size (random collection) and different age groups. All the specimens were excavated from the soil and were in a dormant condition (aestivation).

M. anguillicaudatus is very hardy (Kuhn *et al*., 2024) and can survive out of water for longer durations. To check this quality, specimens were left out of water in a paper cup at room temperature (25˚C) and 90% humidity. The fish survived for 59 h and 35 min. Similarly, a second specimen survived for more than 40 h while a third specimen survived for 39 h. Dead specimens were again shifted to freshwater but none of the specimens revitalized. The decrease in survival time was due to the fact that the other two specimens remained in water for more time than the first one and there was a decrease in temperature and humidity with the passage of time, so they survived for lesser time.

The proportions and ratios have been calculated from the means of observations and measurements and this is due the fact that the specimens whose measurement were obtained for morphometry and hematology belong to different age groups considering their lengths, as well as including all the three collections, so they were heterogeneous in nature. Wide range of deviation from mean for various parameters is also due to the disparity in different age groups.

In standard I, all the 15 observations were similar or (100%) corresponding with the standard for *M. anguillicaudatus* while only one character SL in which the SD was somewhat high but it is to be noted that the collection was random and even this deviation was also present in the standard which was taken. When the Standard deviation was considered then all the values fall in the range of reference standard of that of *M. anguillicaudatus.*

Fowler (1924) and Nichols (1925) work was adapted as standard II for identifying the species under consideration in the current study. Here the one striking character i.e. the Caudal Fin Length was slightly greater than Head Length (HL), a necessary character for *M. anguillicaudatus* (Nichols, 1925). A total of nine parameters were taken here among which seven were similar with that of Fowler (1924) and Nichols (1925) while two characters deviated (Table 4) a little bit which may be due to variations or hybridization as hybridization between pond loaches commonly occur and has been observed in wild individuals of pond loaches. Rare hybridization between pond loach and large scale loach was also observed in one of the wild individuals in Wuhan area, with 74 chromosomes (You *et al*., 2009). The 2nd reason is that in Asia the taxonomy of genus *Misgurnus* and their species morphology overlaps each other (Jakovlic *et al*., 2013). When this data was taken in percent, 78% characters fall in Fowler (1924) and Nichols (1925) for *M. anguillicaudatus* while 22% deviated. The meristic counts of the specimens were all same with that of Fowler (1924) except for specimen 9 (Table 5) whose meristic count for anal fin was same with reference standard but in dorsal fin one ray was more than normal. This ambiguity may be due to genetic variation or some other reason like environmental effects. The scales ranged from 140-155, similar to reference standard as according to Fowler (1924) number of scales range from 138-170 in *M. anguillicaudatus.*

The standard III for our data analysis was a simple correlation formula by Koizumi *et al*. (2007). The formula was applied on the data obtained from specimens and the result i.e. (Y= -0.3) was less than zero which fall in Dojo loach (*M. anguillicaudatus*). This further confirms the inference from first two standards.

The results of hematology were similar with that of Gao *et al*. (2007) for diploid *M. anguillicaudatus*, except for RBC's nuclear length which was little bit larger than diploid (Table 8). The probable reason for this deviation might be due to the dividing state of chromosomes or variations due to diet used. But it is to be noted that these deviations occur from individual to individual not only in fishes but even in human beings also. Still as all the blood cells measurements fall in the range of diploid *M. anguillicaudatus* except for the RBCs nuclear length so the species should be taken as diploid *M. anguillicaudatus.*

Table 8: Measurement of blood cells in diploid (2n), triploid (3n) and tetraploid (4n) *M. anguillicaudatus* (Gao *et al*., 2007**).**

	2n	3n	4n
RBC nuclear length	4.66±0.34	5.47±0.46*	6.50±0.50
RBC nuclear width	3.01±0.11*	3.50±0.31	3.69±0.26
Thrombocytes length	6.81±0.87*	7.65±1.02	8.89±0.99
Thrombocytes width	5.85±0.86*	6.82±0.93	7.69±1.14
Neutrophils length	12.60±1.46*	13.54±1.56	14.88±1.46
Neutrophils width	11.29±1.16*	12.83±1.06	14.08±1.11

* The bold values show coincidence with the standard.

Table 9: Comparison of density of blood cells of old and freshly excavated fishes.

Blood cells	Freshly excavated	Old excavated
Erythrocytes	Less dense	Denser
Neutrophils	Higher	Less
Thrombocytes	Scarce	Higher

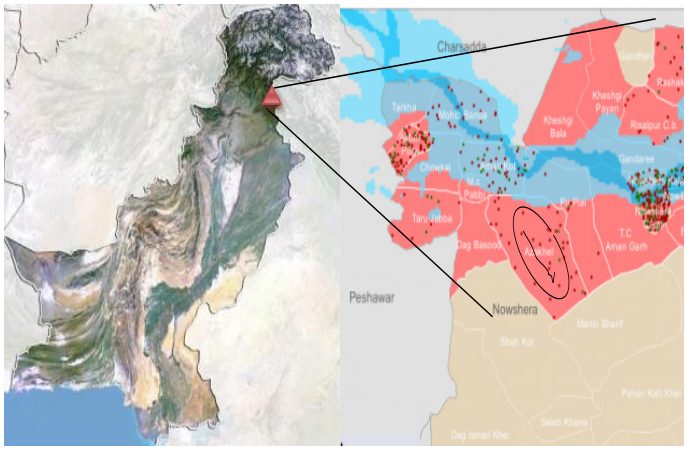

Figure 1: Left side figure shows the map of Pakistan while the right-side figure shows the map of Azakhel (Nowshera). The black circle marks the area where fish was found while the blue color water cannel shows the flood water flowed to the area in 2010

Source: http://www.maps-streetview.com/img/pakistan-satellite-view.jpg; http://img.static.reliefweb.int/sites/reliefweb.int/files/styles/attachment-large/public/resources-pdf-previews/19455-BCCFA3D-06CBD661B8525777B006A57FE-map.png

Figure 2: Ventral view with lateral spots on body.

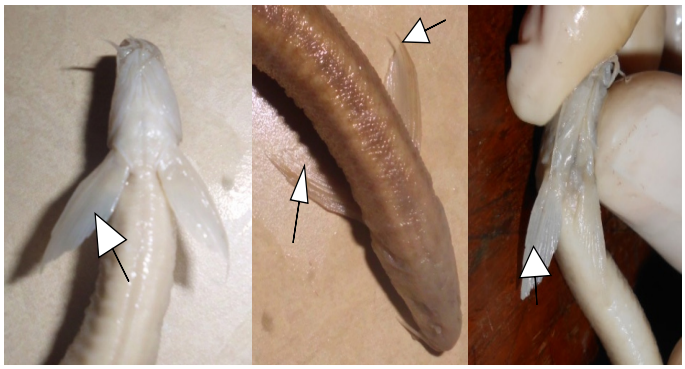

Figure 3: Longest 2nd ray in ♂ in figure 1 and 2 while last figure shows the 2nd ray in ♀ which don't cross the other rays.

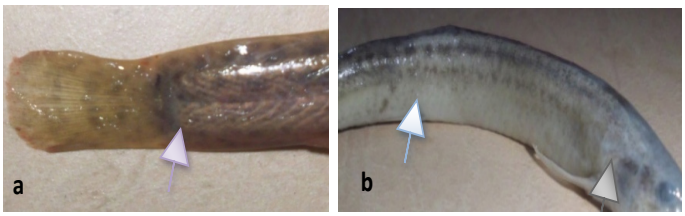

Figure 4: a: Caudal spot; b: Lateral bars on lateral side of the body.

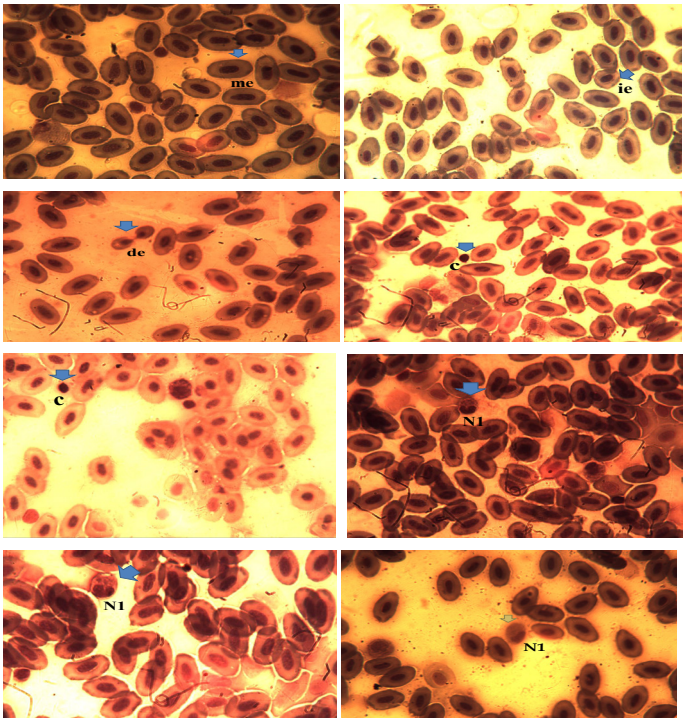

Figure 5: Mature erythrocytes (me), Immature erythrocytes (i.e.), dividing erythrocytes (de) Thrombocytes (c), Neutrophils (N1).

Furthermore, the fish specimens under consideration had the basic characters of *M. anguillicaudatus i.e.* Dark irregular spots on the body, Low adipose crest dorsoventrally on caudal peduncle, and presence of *Lamina circularis* and a small black spot on the upper part of the caudal peduncle. The caudal spot was very small but it is to be noted that the population of these fishes might be isolated geographically from other fish population (*M. anguillicaudatus*) of the world and also the Asian weather loaches show variation up to great extent (Kessel *et al.*, 2013), so probably due the above reasons and environmental differences the spots were not so much significantly visible for identification. Mid lateral stripe from eye to caudal base was absent. The males were with larger and thick 2nd pectoral fin ray (along with lamina circularis) and also had 10 barbels. The dorsal view of the fish was mottled with darker greenish-gray to dark brown marking against brown skin background. All these characters are similar to that of Kessel *et al.* (2013) for *M. anguillicaudatus*. The back and upper surface was distinctly spotted as compared to lower surface with more asterisks posteriorly as shown in (Figure 2). The spots in mature specimens were arranged in two longitudinal shady

or dim lines. The caudal fins of many specimens were adorned with wavy like dusky spots with a black triangular spot broadening toward the upper side never larger than eye. The spots in mature specimens were arranged in two longitudinal shady or dim lines. The caudal fins of many specimens were adorned with wavy like dusky spots with a black triangular spot broadening toward the upper side never larger than eye.

An important question regarding introduction of this fish species in Pakistan is debatable. It has never been reported in any survey from Pakistan. No specimen is present even in Pakistan Museum of Natural History, Islamabad. The arrival of this fish may either be attributed to aquarium trade or its introduction through River Indus. River Indus might have got the fish from Tibet stream (China), which have the concerned species. The 2010 flood in River Indus flowed all over the areas of Nowshehra (study area) and this species may have got a chance to take a refuge in the drainage of Aza Khel Botanical Garden. A thorough survey of the adjoining rivers and streams of the study area and molecular genetic studies of the fish species might answer the question of its introduction in Pakistan.

Conclusions and Recommnedations

In standard I, the result was 100% coinciding with the standard. In standard II the result showed 77.77% coincidence with the reference standard. Likewise, the value of "Y" which is "-0.3" fall in the range of Dojo loach in standard III. In standard IV; the result of hematology also lies in diploid *M. anguillicaudatus*. Moreover, the morphological and other main distinguishing characters along with the number of scales and fin rays arrangement were falling in the range of *M. anguillicaudatus*. Therefore, on the basis of all these observations it is concluded that the species found is Diploid *M. anguillicaudatus.* It is also important to note that the species has never been reported from Pakistan; hence this is its first report in the country. Further studies are suggested not only in other parts of the province but in the whole country as this species being very hardy and adaptive may become invasive and cause serious damage not only to local fish and macro-invertebrate fauna but to the whole aquatic ecosystem as well.

Declarations

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IRB approval

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Ethical statement

Not applicable.

Declaration of generative AI and AI-assisted technologies in the writing process

No generative AI and AI-assisted technologies were used in the writing process.

Conflict of interest

The authors have declared no conflict of interest

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