

Mitochondrial ATPase 6/8-Based Assessment of Genetic Diversity and Population Structure in *Cirrhinus mrigala* Across the Indus River System, Pakistan

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SUMMARY

This study used the sequences of two mitochondrial genes, ATPase 6 and ATPase 8, to analyze the genetic diversity and population structure of *Cirrhinus mrigala* from four geographically distinct river basins in Pakistan's Indus River System. Following the collection of 20 samples from the Taunsa, Chenab, Jhelum, and Ravi rivers, DNA was extracted, amplified by PCR, and sequenced. Genetic heterogeneity was observed among individuals and populations, as sequence alignment revealed a few nucleotide variations in the ATPase 6 and ATPase 8 genes. Phylogenetic analyses showed that the Taunsa and Chenab samples appeared to form a population-specific group. However, the genetic relationships among the populations were very close, suggesting a common origin. High genetic similarity and gene flow across geographically dispersed groups were demonstrated by low genetic distances between many samples. In summary, the findings indicate that *C. mrigala* has comparatively little population differentiation but substantial genetic variation within populations. This information can be useful for comprehending population connectivity, genetic conservation, fisheries management, and the sustainable use of this economically significant fish species in Pakistan.

Keywords: Pakistan, ATPase 6/8, genetic diversity, *Cirrhinus mrigala*, mtDNA

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INTRODUCTION

The cultivable freshwater fish *Cirrhinus mrigala* (mrigal) is a member of the family Cyprinidae (Raza et al., 2022; Arockia et al., 2023). Mrigal is found in every major river system in Bangladesh, Myanmar, India, Pakistan, Thailand and Laos. It is one of the most common species in India, along with other Indian carps cultivated in polyculture systems (Dahanukar). After a rather quick rise up to 1996, with peaks of 552,000 tons in 2000 and 445,000 tons in 2001, the total global mrigal output was pretty constant between 1996 and 2003. However, a comparison of survey data reveals a declining tendency in the catch fishery for this species (Das et al., 2018; Pradhan et al., 2026). Wild populations of Indian carp species are at risk of losing

genetic variety and variability due to a decline in genetically distinct wild stocks and mixing with farmed accidental escapes or reservoir stock programs (Das et al., 2018).

The selection of founder stock for breeding programs is based on the genetic heterogeneity of natural stock. Thus, knowledge of genetic variability data would benefit the establishment of genetic development initiatives, efficient controlling, and protection of natural genetic resources. DNA markers are essential instruments that have been utilized to assess the patterns and levels of genetic variation in several cultured fish species (Pradhan et al., 2026). Among the variety of DNA markers currently available, mitochondrial DNA (mtDNA) has been used extensively as an effective marker system due to several beneficial features, such as a fast rate of mutation that makes it useful for detecting recent population isolation, establishing genealogical relationships among populations within species, and for evolutionary genetics and species identification (Palomo-Díez and López-Parra, 2022; Elyasigorji et al., 2023).

One important species of freshwater fish frequently found across Pakistan's Indus River system is *Cirrhinus mrigala* (Altaf et al., 2011b; Altaf et al., 2015a; Ashraf et al., 2022; Iqbal et al., 2023; Syed et al., 2026). Despite its ecological and commercial significance, little is known about the genetic diversity and population structure of this species across different regions. Gene flow between populations may be impacted by habitat loss, excessive exploitation, river fragmentation, and human-caused disturbances, potentially reducing genetic diversity. To ensure sustainable fish supplies, improve aquaculture development programs, and establish effective conservation methods, it is essential to comprehend population genetic structure. Therefore, a better understanding of the genetic state of *C. mrigala* populations in Pakistan's Indus River system requires an assessment of genetic variation and population differentiation utilizing mitochondrial ATPase 6/8 gene sequences.

MATERIALS AND METHODS

STUDY AREA

The Indus is a trans-Himalayan river in South and Central Asia, as well as a transboundary river in Asia. The 3,180 km river, known as Sengge Zangbo, originates in the Tibet Autonomous Region of China. It flows northwest through the disputed Kashmir region, first through the Indian-administered Ladakh and then the Pakistani-administered Gilgit-Baltistan. After bending sharply to the left after the Nanga Parbat massif, it flows south-by-southwest through several Pakistani provinces before splitting and emptying into the Arabian Sea, with its main stem close to the port city of Karachi (Rahman et al., 2021; Rahman et al., 2023; Syed et al., 2026).

In the Lahaul and Spiti region of the Indian state of Himachal Pradesh, at Tandi, 8 km southwest of Keylong, two rivers, Chandra and Bhaga, converge to form the Chenab River. Surya Taal Lake, located in Himachal Pradesh a few kilometers west of the Bara-lacha La Pass, is the source of the Bhaga River. East of the same pass, glaciers give rise to the Chandra River. Additionally, this pass serves as a water split between these two rivers. Before coming together at Tandi, the Bhaga River travels 60 km through small gorges, and the Chandra River travels 115 km. The Chenab River's bank is in Multan, Punjab, Pakistan, and the Trimmu Barrage, where the Jhelum River empties into the Chenab, is situated in Jhang, Punjab, Pakistan.

After passing through the Pangi Valley in the Chamba district of Himachal Pradesh, the Chandra-Bhaga enters the Jammu division of Jammu and Kashmir. The two rivers converge at Sansari Nallah, which is situated in the final zone of the Paddar region of the Jammu division. The districts of Kishtwar, Doda, Ramban, Reasi, and Jammu are traversed by it. The Panjnad River is created when it empties into the Sutlej after entering Pakistan and passing through the province of Punjab (Altaf et al., 2008; Altaf et al., 2011a; Altaf et al., 2014; Altaf et al., 2015a; Altaf et al., 2015b; Altaf, 2016; Altaf et al., 2017; Umair et al., 2019; Altaf et al., 2020; Altaf et al., 2023; Batool et al., 2026).

SAMPLING

Twenty *Cirrhinus mrigala* blood samples were taken from several locations in Pakistan's Indus Riverine System, including Head Qadirabad (River Chenab), Head Rasool (River Jhelum), Head Balokki (River Ravi), and Head Taunsa (River Indus). The caudal vein was punctured to get blood, which was then stored in vacutainers. After that, the samples for DNA extraction were kept at -20°C in the Institute of Biochemistry and Biotechnology (IBBT) laboratory at the University of Veterinary and Animal Sciences in Lahore (Figure 1).



Figure 1: Map of the study areas.

GENOMIC DNA EXTRACTION

Genomic DNA was extracted using an inorganic technique. The intended product was subsequently amplified using PCR with this DNA. The methodology was altered to achieve the intended outcomes since fish red blood cells are nucleated. Samples of blood were thawed at 37 °C in a water bath. After taking a 200 µL sample of blood, it was thoroughly mixed with 1 mL of lysis buffer by inverting and vortexing. The resulting mixture was centrifuged for 15 minutes at 4 °C at 12,000 rpm. After that, the supernatant containing RBCs and other blood components was discarded. The pellet was then treated with 100 µL of ten percent SDS, 80 µL of proteinase K, and 1 mL of Buffer A (Sambrook and Russell, 2001).

To break down the protein, it was thoroughly shaken and left in a water bath at 55 °C for the entire night. 350 µL of PCI was added to the tubes during an overnight incubation period. After thoroughly mixing the tubes, they were kept at a low temperature. The samples were centrifuged once more at 4 °C for 15 minutes at 12,000 rpm. Two of the three layers that emerged in the tube this time were the most noticeable. DNA in soluble form was found in the upper aqueous layer, while contaminants were found in the lower layer.

Using a pipette, the supernatant was extracted into fresh tubes. The pellet was discarded, and the fresh tubes were given the same label. After adding an equal volume of isopropanol to the tube, it was carefully inverted until DNA threads were visible. For twenty minutes, the tubes were left at room temperature. The tubes were centrifuged for 15 minutes at 4 °C at 11,000 rpm. The pellet remained after the supernatant was disposed of. Seventy percent ethanol was used to wash the DNA pellet. After centrifuging the samples for 20 minutes at 12,000 rpm, the pellet was left behind and the ethanol was discarded. The DNA pellet was stored at room temperature until the ethanol odor subsided. Double-distilled water and a quick spin were used to dissolve the DNA. To enable thorough mixing, the DNA was maintained at ambient temperature. The nucleases were then rendered inactive by a 30-minute heat shock at 70 °C in a water bath. Lastly, the DNA was maintained in containers with unique labels and kept in a freezer at -20 °C.

DNA QUANTIFICATION

DNA was diluted in TAE buffer. 1.0 µL of the extracted DNA was pipetted onto the Nanodrop pedestal, and a reading was taken. The reading was displayed on the computer, and the concentration (in ng/µL) and A260/A280 ratio were recorded (McLean, 2021).

QUANTIFICATION OF DNA BY GEL ELECTROPHORESIS

A 0.8% agarose gel was used for DNA quantitation (Slater et al., 1988). This is how the gel was made: In a conical flask with 100 ml of 1× TAE buffer, 0.8 g of agarose was dissolved. The flask was placed in a microwave oven for one minute. After removing the solution, it was allowed to cool to ambient temperature. To visualize DNA, 8 µl of ethidium bromide was then added to the mixture. To avoid bubble formation, the agarose and ethidium bromide were carefully combined and then added to a gel casting tray. After that, the gel was allowed to solidify at room temperature. Each well was filled with a mixture of 2 µl of DNA sample and 1 µl of

6X bromophenol blue loading dye. A voltage of 120 V was applied for half an hour. A Bio-Rad gel documentation system was used to visualize the bands under UV light. Dilution was used to bring all samples to the same concentration. Stock DNA was diluted to a concentration of 25 ng/μl using a NanoDrop and gel imaging.

PRIMER DESIGNING

Using the online Primer3 program (<http://primer3.ut.ee>), customized primers for *Cirrhinus mrigala* created for the PCR amplification of ATPase 6/8 genes (Kõressaar et al., 2018).

PCR AMPLIFICATION FOR RANDOM SEQUENCES FROM FISH SAMPLES

Primers were used to create the polymerase chain reaction (Sevilla et al., 2007). Every reaction was carried out in 0.2 mL tubes that held 25 μL of the reaction mixture. To make a 25 μL reaction mixture, mix 2.5 μL of 10x PCR buffer, 2 μL of 2.5 mM dNTPs, 1.5 μL of MgCl₂, 0.10 pmol/μL primer, 1 μL of DNA, 0.3 μL of 5 units/μL Taq polymerase enzyme, and 16.7 μL of sterilized deionized water. Sterilized water was used as the template for each reaction's negative control.

PROFILE OF THE PCR REACTION

A thermal cycler (Bio-Rad T100, USA) was used to conduct the PCR process (Hayden et al., 2013). The conditions for the amplification of ATPase 6/8 were as follows: one cycle of three minutes for denaturation at 95°C, followed by thirty seconds at 94°C, thirty seconds for annealing at 57°C, and thirty-six seconds for extension at 72°C. The machine was kept at 4°C.

ANALYSIS OF PCR PRODUCTS

A 1.2% agarose gel was used to evaluate each PCR result. 1.2 g of agarose was dissolved in 100 ml of 1X TAE solution to create the agarose gel. DNA loading buffer was used to load DNA samples into wells. To measure the sizes of the samples, a 1 kb ladder was run alongside them. A gel documentation system (Bio-Rad, Gel-DOC EZ Imager) was used to capture pictures and visualize the gels under UV light.

AGAROSE GEL ELECTROPHORESIS

1.2% agarose gel electrophoresis was used to analyze amplified DNA products (Zoetendal et al., 1998). DNA samples were combined with loading dye and then placed onto the gel. The amplicon's size was verified using standard molecular weight markers. The gel was run in Tris-borate-EDTA buffer at a steady voltage. Ethidium bromide solution was used to stain the gel, which was then observed under a UV lamp and recorded using a gel documentation system (Syngene, GeneGenius Bio Imaging).

SEQUENCING OF ATPASE 6/8

An ABI 3130XL genetic analyzer was used to perform dideoxy chain termination sequencing after specific amplicons were purified by ethanol precipitation (Zafar et

al., 2020). Salts were eliminated by washing with 70% ethanol after the intended DNA template was purified by precipitation with pure ethanol.

STATISTICAL ANALYSIS

The obtained DNA sequences were analyzed using various bioinformatics tools. Multiple sequence alignment was performed using Clustal W and BioEdit software, and phylogenetic analysis was conducted using MEGA 5.2 software. While Past Software was also used to calculate the ANOVA.

RESULTS

Separating on an agarose gel was used to evaluate each PCR result. As mentioned in the preceding section, a 1.2% agarose gel was made in TAE buffer for this purpose. After that, DNA loading buffer was used to load the DNA samples onto the gel. A 100-bp DNA ladder was used in one or both outer lanes of each gel. A gel documentation system (Bio-Rad) was used to take pictures and visualize the gels under UV light.

Every DNA sample was gathered from several areas within Pakistan's Indus Riverine system. The phylogenetic examination of the selected species was carried out using MEGA 6.0 software. Although there was substantial diversity among the fish samples, phylogenetic analysis revealed that they all shared a common ancestor. Figures 2 and 3 show a branch length of 0.05. These consequences suggest that all taxa share a common ancestry and a significant relationship among all DNA samples collected from various locations. Tables 1 and 2 show the alignment of sequence variations in samples with reference sequences. At position 466, C is replaced by T in sample 2-6. At position 9, A is replaced by C in sample 13-6, and T is replaced by C in sample 5-8. A is replaced by C in sample 13-8. T is replaced by C in samples 12-8 and 19-8.

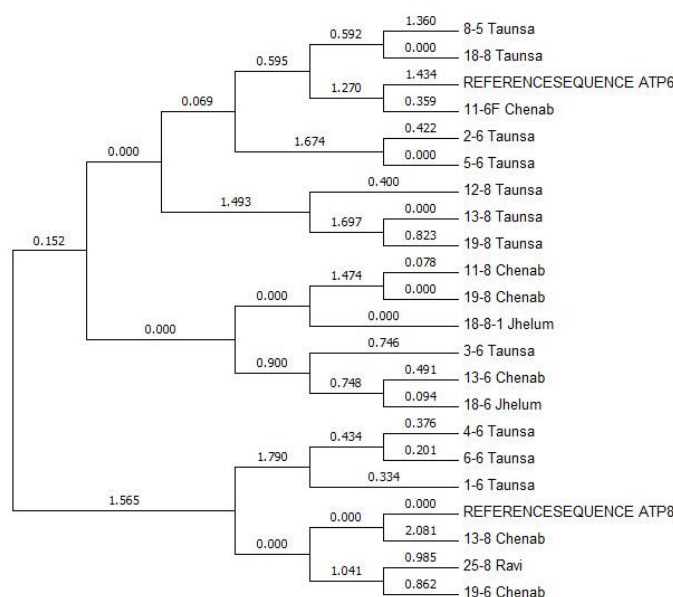


Figure 2: Phylogenetic Tree of Local species with Reference sequence

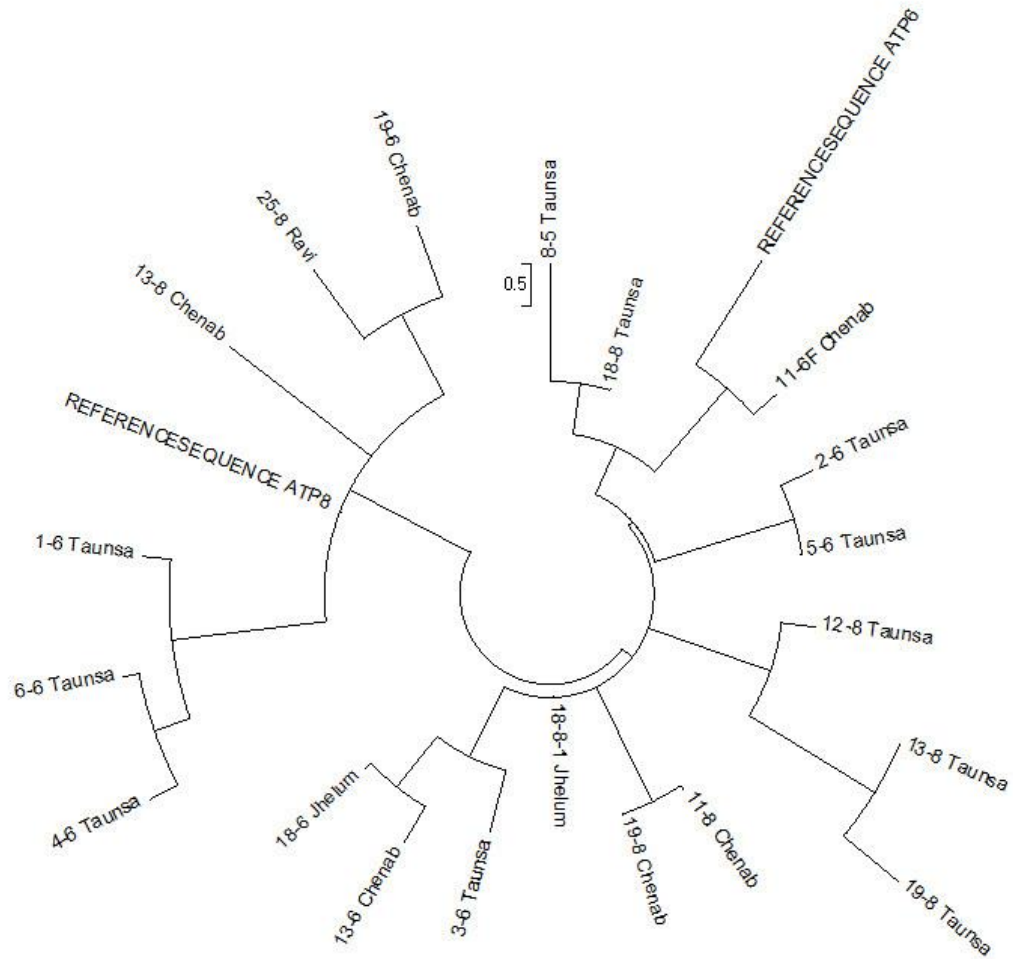


Figure 3: Phylogenetic Tree of Local Species with Reference Sequence (Circular view).

MEGA 6.0 was used to build the NJ tree. There was a 0.05 branch distance. The size of the ATPase 6 gene fragment was 690 bp. The findings indicated that various fish species share a common ancestor. MEGA 6.0 was used to create the NJ tree for the ATPase 8 gene. There was a 0.05 branch distance. The size of the ATPase 8 gene fragment was 290 bp. Common ancestry was revealed by the results. The Chenab samples are more closely connected to one another, according to the results. Although the Chenab samples are more closely connected to other Chenab samples than to those from Jhelum or Ravi, they are nonetheless related to both. Samples from Taunsa are more closely connected to one another.

The NanoDrop DNA concentrations of the sampled geographical populations did not differ significantly, according to the Kruskal–Wallis test ($H = 1.762$, $p = 0.4143$), suggesting that the groups' median DNA concentrations were statistically comparable (Table 3).

Table 1: Alignment of Sample Variations with Reference Sequences (ATPase 6 Gene).

Position on Reference sequence	601	466	477	656	9	594	535	376	186
Reference Sequence	G	C	A	A	A	C	-	-	T
1-6 Taunsa	-								
2-6 Taunsa		T							
3-6 Taunsa			-						
4-6 Taunsa			-						
5-6 Taunsa				-					
6-6 Taunsa				-	-				
11-6 Chenab				-					
13-6 Chenab					C				
18-6 Jhelum				-				A	
19-6 Chenab				-				C	

Table 2: Alignment of Sample Variations with Reference Sequences (ATPase 8 Gene)

Position on Reference Sequence	9	8	21
Reference sequence	T	A	T
8-5 Taunsa	C		
11-8 Chenab		-	
12-8 Taunsa			C
13-8 Chenab		C	
13-8 Taunsa			C
18-8 Taunsa		-	
18-8-1 Jhelum		-	
19-8 Chenab		-	
19-8 Taunsa			C
25-8 Ravi		-	

DISCUSSION

This work has shown that direct sequencing of variable mtDNA segments can yield valuable information about the phylogeographic structure and genetic divergence of fish populations. Here, we used partial ATPase 6/8 genes sequencing to examine the stock structure of *Cirrhinus mrigala*. It explains the general genetic difference between and within *Cirrhinus mrigala* communities that live in 4 rivers along Indus River. The ATPase 6 (Swain et al., 2016) and 8 gene's nucleotide base representation order, A>C>T>G and A > T > C > G, are consistent with data for Indian major carps (Habib et al., 2012). All of the mtDNA protein-coding genes of Indian major carps had the lowest representation of nucleotide 'G' (Habib et al., 2012; Das et al., 2018). The sequence alignment revealed several nucleotide polymorphism areas in both genes, indicating genetic variation across individuals from the four sampling locations (Taunsa, Chenab, Jhelum, and Ravi) as well as those from other sampling sites. The ATP6 gene showed nucleotide frequencies in the pattern A>C>T>G, indicating that guanine (G) was the least common nucleotide, which is consistent with a previous study on Indian big carps. Several locations, including 601, 466, 477, 656, 9, 594, 535, 376, and 186, exhibited variation in ATP6, whereas positions 9, 8, and 21

showed variation in ATP8. Multiple mitochondrial haplotypes may result from nucleotide variations and alignment gaps. Eleven haplotypes were discovered in 155 specimens in the previous study (Das et al., 2018); the majority of them were shared between populations, but others were population-specific, suggesting gene flow and population differentiation (Bej et al., 2013; Das et al., 2018). Similarly, genetic linkage and differentiation across the *C. mrigala* populations of the Indus Riverine System might be evaluated using the common and unique nucleotide sequences found in this study.

The phylogenetic tree shows that *Cirrhinus mrigala*'s ATP6 and ATP8 sequences form multiple distinct clusters (Katyal et al., 2021). Some individuals establish close clusters with individuals from different geographic populations, while others form close clusters with individuals from the same geographic population. Low genetic distances, such as 0.000, are found in a few samples, suggesting that the sequences are almost identical or extremely similar. More diverse sequences are indicated by higher genetic distances. With the ATP8 reference sequence tending to form a distinct, lower cluster from the majority of ATP6 samples, the overall tree indicates genetic closeness and considerable differentiation among the sampled populations. The phylogenetic tree shows that *Cirrhinus mrigala*'s ATP6 and ATP8 sequences form multiple distinct clusters. In fact, some of the genetic distances found in our samples were as low as 0.000, which is consistent with the low nucleotide diversity previously described, indicating little overall mitochondrial differentiation and a high degree of similarity. According to the earlier study, an F_{ST} of 0.02466 indicates that there is minimal genetic variation between groups, with 97.53% of genetic variation found within populations and 2.47% between them. Because of this, the phylogenetic tree's overall clustering pattern supports the earlier finding that *C. mrigala* populations can have high levels of gene flow and a shared genetic ancestry. Certain clusters and genetic distances also point to potential population-specific evolutionary histories and local genetic differentiation. The findings are similar to those of Das et al. (2018), who showed 97.53% of the genetic variation within the populations, low nucleotide diversity (0.00154), and low overall genetic differentiation ($F_{ST} = 0.02466$). While a few separate groupings may imply little heterogeneity between the populations, the clustering of individuals from several geographic populations may show similar ancestry and gene flow. Overall, our findings confirm that *C. mrigala* populations exhibit modest genetic differentiation and strong genetic connectedness.

The Kruskal–Wallis test was used to determine if there was any significant difference in DNA concentrations, as measured by the NanoDrop, between the geographical populations sampled for *Cirrhinus mrigala*. The analysis revealed no statistically significant difference between the groups. The null hypothesis that the population medians are equal was not rejected ($p > 0.05$). As such, the differences in DNA concentration between the various sampling locations were not statistically significant, suggesting that the amount of extracted DNA was fairly similar across populations. This indicates that variations in DNA concentration were not likely to significantly impact subsequent PCR amplification and sequence analysis.

CONCLUSION

This work demonstrated the value of mitochondrial ATP6 and ATP8 sequences in assessing population relationships and genetic diversity in *Cirrhinus mrigala* from the Indus River System. Although a few discrete clusters indicated little population-level differentiation, the phylogenetic analysis revealed generally close genetic relationships among populations. Significant genetic resemblance and possible gene flow between geographically separated populations are implied by the existence of very low genetic distances. Overall, the findings show that the populations under investigation have a similar genetic heritage and relatively little genetic difference. These results provide important baseline information for *C. mrigala* population management, conservation, and genetic assessment in Pakistan.

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Table 3: Number of samples with given identity and nanodrop concentration.

Sr.	Sample Number	Given Identity	Nano-Drop concentration(ng)
1	Sample 1	5-8 Taunsa	500
2	Sample 2	11-8 Chenab	500
3	Sample 3	12-8 Taunsa	600
4	Sample 4	13-8 Chenab	600
5	Sample 5	13-8 Taunsa	700
6	Sample 6	18-8 Taunsa	500
7	Sample 7	18-8 Jhelum	400
8	Sample 8	19-8 Chenab	500
9	Sample 9	19-8 Taunsa	456
10	Sample 10	25-8 Ravi	600
11	Sample 11	1-6 Taunsa	700
12	Sample 12	2-6 Taunsa	650
13	Sample 13	3-6 Taunsa	560
14	Sample 14	4-6 Taunsa	570
15	Sample 15	5-6 Taunsa	500
16	Sample 16	6-6 Taunsa	480
17	Sample 17	11-6 Chenab	600
18	Sample 18	13-6 Chenab	420
19	Sample 19	18-6 Jhelum	520
20	Sample 20	19-6 Chenab	630

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