

Academic Journal of Nawroz University (AJNU), Vol.11, No.4, 2022 This is an open access article distributed under the Creative Commons Attribution License Copyright ©2017. e-ISSN: 2520-789X https://doi.org/10.25007/ajnu.v14n11a1194



# Molecular Identification and Phylogenetic Relationship of Some Plant Species In Ashdagh Mountain, Sangaw, Kurdistan Of Iraq

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# ABSTRACT

Phylogenetic and molecular perspective of plant identification can reveal the plant composition of areas. Many new species have been identified based on morphological characters in different area of Kurdistan, but few have been identified through DNA barcoding method. In this study, Internal transcribed spacers 1 and 2 (ITS1 and ITS2) were used to identify some species in Ashdagh Mountain. The identified taxa were belonged to different families and clades and not recorded in any studies. The nucleotide sequences of identified species were 98-100% identical with species in the genebank database. The phylogenetic analysis showed diversity in phylogenetic relationships among coexisting species. In addition, the existing of Chara sp. in the acidic stream of Awa spi is a new record and not observed before. The finding of this study show the importance of using molecular and phylogenetic technique to identify and understand phylogenetic relationship among coexisting species occurrence data has been added to flora of Kurdistan.

KEY WORDS: Phylogenetic, DNA barcoding, ITS, Chara, Kurdistan.

# 1.

Molecular based species classification is considered an accurate method to identify species (Almerekova et al., 2020; Li et al., 2015). It has ability to tease out the intraspecific variation among more morphologically resemble species (Álvarez & Wendel, 2003). In addition, looking at the genetic sequences will help to understand how species evolve (i.e. convergent or divergent evolutions) (Ray, 2014). Recently, the taxonomic placement of many species have been changed after doing molecular analyses for them. The use of molecular data enables taxonomists to classify species at species level using DNA barcoding (short DNA sequence)(Hebert, Cywinska, Ball, & deWaard, 2003). Internal transcribed spacers 1 and 2 (ITS1 and ITS2) are part of ribosomal DNA. They are consider important DNA markers in molecular plant identification (Cheng et al., 2015; Li et al., 2015; Mishra et al., 2016; Qin et al., 2017). ITS regions are part of rDNA (ribosomal DNA) and existing between 18S and 28S genes. They have high evolution rates and

# Introduction

easy to amplify in the lab (Coleman, 2007). These characteristics enable the taxonomists to use them to identify the species within and among taxa and endangered species (Hebert *et al.*, 2003; Hollingsworth, Graham, & Little, 2011). In the phylogenetic analysis, the use of ITS sequences will tell us the accurate placement of species along phylogenetic tree of coexisting species in certain area (Zhu *et al.*, 2018).

In Kurdistan, most of the classification studies were done based on morphological characteristics. So, incorporating of molecular phylogeny using DNA barcoding into species identification and classification processes will help identify and classify species more accurately than relying only on morphological identification (Nilsson *et al.*, 2008; Yao *et al.*, 2010).

The aim of this study is to do molecular identification for some species in the Sangaw area, Kurdistan. Results of this study will serve as a field guide for researchers to do more taxonomical, ecological and physiological studies on plant composition structure since there are limited or no floral classification studies done in this area. Especially, the area characterized by distinct geological and geographical characteristics which make it more suitable for high floral diversity (Saman *et al.*, 2013; Sami *et al.*, 2019).

#### 2. MATERIAL AND METHODS

#### 2.1 Study area and sample collection

Sangaw is a subdistrict of Chamchamal district and it located in the northeastern part of Iraq (35.2851° N, 45.1799° E), about 35 km from southwest of Sulaimani city and 6 km west of Qara Dagh. More specifically, west of Sagerma Mountain. The region characterized by distinct geological structures (Kharajiany, 2013). A total of 18 taxa, representing 17 plant species and one algal species (Table 2), were collected from different locations on Ashdagh mountain, while the algal sample was collected from downstream of Awaspi river. The stream is an acidic stream, and it running through Darzilla village, Sangaw. The samples were collected on February 2-5, 2021. The leaves samples (n=10 leaves/plant) were stored in a zipper bag and transferred to the lab for molecular analysis (Polymerase chain reaction).

# 2.2 DNA extraction, amplification, and sequencing

AddPrep Genomic DNA Extraction Kit was used to extract the total genomic DNA. Two universal nuclear regions named internal transcribed spacers 1 and 2 (ITS1 and ITS2) primers were used to identify the unknown species and do phylogenetic analysis. These two primers are widely used in plant phylogenetic studies due to their ability to teasing out the intraand interspecific variation among species (Almerekova *et al.*, 2020; Li *et al.*, 2015). Primers used in this study are listed in Table 1.

In the standard PCR, each reaction contained (MyTaqTM HS Mix-Bioline, USA) master mix (10  $\mu$ L) 10 pmol of each primer and 20ng template DNA in a final volume of 20 $\mu$ L. PCR was performed using a

three-step cycling protocol: initial denaturation (95°C/5 min) 1 cycle; [(denaturation (95°C/ 30sec), annealing (57°C/ 30 sec) (extension 72°C/ 30 sec) 40 cycle] and final extension (72°C/5) 1 cycle (Bio-Rad C1000 Thermal Cycler, USA). The PCR products were analyzed in 1% agarose gel (Only 1g of agarose was dissolved in 100 mL (1X TAE buffer) TAE: Trisacetate EDTA) stained with Ethidium bromide (Figure 2).

Table 1. Universal plant ITS primers (Cheng *et al.*, 2015)used in this study.

	5
DNA	Sequences (5'–3')
markers	Forward primers
ITS1	GGAAGKARAAGTCGTAACAAGG
ITS2	CAWCGATGAAGAACGYAGC
	Reverse primers
ITS1	GCGTTCAAAGAYTCGATGRTTC
ITS2	RGTTTCTTTTCCTCCGCTTA

**2.3 Blast, sequence alignment, and phylogenetic tree** Forward and reverse sequences of internal transcribed spacers regions per species have been edited and combined using Bioedit software (Hall, 1999). Nucleotide blast (Blastn) function in NCBI (National Center for Biotechnology Information) database was used to identify each species. Identification of species was based upon blast (% identity) and distance tree (fast minimum evolution and neighbor-joining) in NCBI online database.

Sequences were aligned using the ClustalW function in Bioedit with some manual adjustments (Figure 2). The length of the combined regions (ITS1 and ITS2) included 1430 base pairs. Mega X (Kumar *et al.*, 2018) was used to build Neighbor-Joining (NJ) tree. The use of both ITS1 and ITS2 in the phylogenetic analysis will provide better understanding of phylogenetic relationship among coexisting species (Qin *et al.*, 2017). The algal species (*Chara sp.*) was selected as an outgroup.

## **3. RESULTS**

The PCR analysis showed clear band of 18 samples

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(Table 2), with a band of 300-500bp corresponding to the amplification of the forward and revers ITS1 and ITS2 sequences. The nucleotide sequences of identified species were 98-100% identical with species in the genebank database. While, other taxa showed low identical percentage (<90%) with other species of same taxa in the database and marked as "sp.". The identified taxon have belonged to 12 families, and most of them are members of the rosids clade (Table The sequence alignments showed significant 2). variation in the base pairs among species (Figure 2). The existing of such variation is due to that ITS regions are not conserved and its subjected serial of addition and deletion of base pairs.



Figure 1: Agarose electrophoresis image, showing the PCR results for the collected species of ITS1 and ITS2 regions at 300-500 bp PCR product size. (A) Forward and reverse sequence of ITS1. (B) Forward and reverse sequence of ITS2. Lane M: Marker 100bp.

Table 2. List of the identified species in this study with

No.	Species	Family	Clade		
1.	Linum		Rosids		
	mucronatum	Linaceae			
2.	Onobrychis		Rosids		
	ptolemaica	Fabaceae			
3.	Xanthium		Asterids		
	strumarium	Asteraceae			
4.	Teucrium		Asterids		
	oliverianum	Lamiaceae			
5.	Euphorbia		Rosids		
	Craspedia	Euphorbiaceae			
6.	Glaucium flavum	Papaveraceae	Eudicots		
7.	Alcea sp.	Malvaceae	Rosids		
8.	Onopordum		Asterids		
	tauricum	Asteraceae			
9.	Reseda aucheri		Rosids		
	subsp. aucheri	Resedaceae			
10.	Alcea pallida	Malvaceae	Rosids		
11.	Umbilicus sp.	Crassulaceae	Core		

			eudicots
12.	Haplophyllum sp.	Rutaceae	Rosids
13.	Verbascum sp.	Scrophulariaceae	Asterids
14.	Thymbra sp.	Lamiaceae	Asterids
15.	Chara sp.	Characeae	Charophyta

	10	20	30	40	50	60	70	80	90	100
lings more			 077			 GACACA_CAT				
Hanlophyll				GITARICCIC	GACOOOGICO	GACACA-GAT	TOOGGAGACA	TTGTCGAACC	TOCCAGCAA	2002000000
Reseda and	CGAGAACAAG	TATTG-TGAT	GCGGAAGCCG	GCAGGGCTCT	GTACG-GACT	GTOGGTTTCC	GATGCCTGCG	GAGGCGCGGG	AGAATCC	CTAGGTTTGC
Alcea sp.	GATATCCA-C	TATTGAATGA	GGGGGTGAGG	ATGCATCTTT	GCCCCCAACC	CCCTCGTTGC	CTAGGTGTGC	TTGGCCTTTC	CTCATTCGTT	CTTGGGCGG-
Alcea pall	GATATCAAAC	AATTGAATGA	GGGGGTGAGG	ATGCATCTTT	GCCCCAAACC	CCCTCGTTGC	CTAGGTGTGC	TTOGCCTTTC	CTCATTCGTT	CTTGGGCGG-
Euphorbia	CGTGAACGTG	TTTGTAAACC	GGGGGG <mark>T</mark> CGG	CGCGGGATTC	GTCCAGTGTC	GGCCCTCCAC	GGGG <mark>CCC</mark> G-G	GCGAGGGGTG	CGGGTGC	GGG <mark>CTC</mark> A
Onobrychis										
Teucrium_p	TGTTTA-	ACC	-AATTCG	GGAA	GG	CGG	CGAGGGGGCT	TOCCCCCT	-TCCCTTGCC	CCAACCCCGC
Thymbra_sp	AGTGACCTGC	GGAGATCATT	GTCGAACTTG	CAAGCAGACC	GCGAACACGT	GTTTTACCAC	TTCGGGGCAG	TUGTAAUUUU	GUGACULTUT	TCCGTGTCCC
Yanthim s	ACCIO		C	GAA	CTOLARGERT COTTACOTT	CONCEPTOR	GAGGCCTT-G	TTGGCGTGTG	TTTATOCTTO	CACCATACAT
Onopordum	CGTGAACACG	TAATCACAAC	CGGGCGTCGG	GGGGATCGG-	GTGTGAGCCC	GGTGCCC-GC	GATGCTCC-G	TCGGCGTGCG	TGCAAGGT-G	CCCCTT-CC-
Umbilicus	AGT-ACCTGC	GGAGA-CATT	GTCGAACCTG	ACAGCGAACC	GCCCAATTTT	GTTT-ACCGG	GTAGTCTCGG	GGATCCCTCG	GGCTCCCCTT	OCCTOCCC
Glaucium_f		AACGAC	CCGCGAACAC	GTCAATCCAC	GTCCGGGGGAC	GCTCCOGAOG	GGGGAGAGAG	ATCTCCCCCC	CTCGGCGCTT	CCCsCGGC
Chara_sp.	AGTACTGCGG	AGACATTGTC	GATCCTGCCT	AGCAAACGAC	CCGCGAACAT	GTTTTCCCGT	CCGGGTACTC	TCCGGAGGGG	GGACTTCTCT	GCCCTTCTTT
	11	120	130	0 140	15	16	170	180	190	200
Linum muer	ATTTGCTCG	-GTCCTAGTG	TCCTTGCCTT	CCCACACAAA	CTAACAAT	CCCCCGACGC	GCATGOGTC	AAGGAATACT	TCGAGCG	AACGTCGC
Haplophyll	AACTCGTG	-AAAATCAC	CGGGGAGGCG	GGGGCCCTCG	TGCCCCCTCC	CCCCCAACCG	GC	-CGCCTT	G	AGCCCTTTGT
Reseda auc	CTGGCGG-	-GGCCCCTCG	CGCTG	-TAAGCAGGA	ACAACAAA	000000000000000000000000000000000000000	GTTAAGCGTC	AAGGAAC <mark>T</mark> GA	AAAGCAAGCG	AACGCGCCAT
Alcea_sp.	GTGA-	-AATTATTTG	TCTTGTTGCA	<b>CTCATGTTAA</b>	AT-GAAC-AA	CCCCCGGCCC	GAACTGCGTC	CAGGAATTAA	AAA <mark>T</mark> GAA <mark>T</mark> AG	AGTGCACGTC
Alcea_pall	GTGA-	-GATTATAGG	TCTTGTTGCA	CTCAGGTAAA	AT-GAAC-AA	CCCCCGGCGC	GAATTGCGTC	AAGGAATTAA	AAA <mark>T</mark> GAAAAG	AGTGCACGTC
Euphorbia_	CCCTTCC-	-CCCTTCACC	CACCGT	CCGTGGCCTC	CTAACAAA	ACCCCGGCGC	COTACOCOCC	AAGGAATTGC	AAACGAAAAG	ACCGCATGCC
Unobrychis Tengrinm r	ACCOMMENCE	0007000000	T_070000. T	000001107		ATTO20000	GAAAGGOOOG	77007477744	CAAAG C	ACCORC
Thumbra sp	ABBGAACTOG GTCTCCCCCC	CCCTCGCCCG		OCCOURT I	CT AACGAA	C-00000000	GRAATGCACC	GAGGAAAACC	AAACT	ACCOTCCC
Verbascum	GCACG	-GCGT-GCGC	ACGAGCGACC	GTCGTGCGTG	CT-AACGAA	CCCCCGGCGC	GGCATGCGCC	TAGGAAAACT	CAACGA	AGCGCCTCCC
Xanthium s	GTGG	GGCA-TCATG	GATTT-CACG	TTGACACACT	AACAAA	CCCCCGGCAC	GGTACGTGCC	AAGGAAAACT	AAACTTAA	AGGGCCCGTG
Onopordum_	GG	GGCA-TCGTG	TGCGT-CCCG	TCGTCACCGA	AACAAA	C-CCCGGCAC	GGCATGTGCC	AAGGAAAACA	AAACTTAAGA	AGGGCGCGTC
Umbilicus	CCATGCCGGC	CGGACCCGTG	CCCCTCTCTT	TGCTTGCGCG	TAACGGC	<mark>CCCGGCGC</mark>	GGATTGCGC-	GAGGAAT	CTTCCAC	GACCCCTTTC
Glaucium_f	CGCCCCGG-	-GCTCTGGCT	TGCCTGCGCC	CGGACGGAAA	AAACGAAC	CCCACGGCGC	GGACCGCGCC	AAGGAAC	AAACAAAAGG	AGAGGGCGCG
Chara_sp.	GTGCCCG-	GGTGATCGG	CGTGGGTGCC	GTCTCGGCCA	TGACCAAA	CCCCCGGCGC	ACGGGGCCTC	CTAAAAC	-ACTITAAGC	AGGGTCCGCG
	21		230	0 240	25	D 26	270	281	) 29( 	300
Linum mucr	GCCTTGT	OCCCOOT-CA	CGGTGCATTG	GGCGGGG	CGA	TTCACCAC	ATAATT	TACTATAACG	ACTCTCOGCA	ACOGATATCT
Haplophyll	CTTTGGT	CTCCCCT	TC		<mark>CGTC</mark> AAA	TTTAA	TAATTAAAAC	TTTCGCCCGG	AC	ATCTCT
Reseda_auc	CCGT	GCCCCGT	TCTCGGGG	<b>TGCGGT</b> GG	CCGCGATGC-	TGTCT	T-CGAATGAC	ACAAACG	ACTCTCGGCA	ACGGATATCT
Alcea_sp.	TACTGTCATC	GTCCCGTAAG	CCATGTT	TGTGTGGCAG	TGACGCTGTT	ACTITITGTCG	TGAA	ATACAAAACG	ACTCTCGGCA	ACGGATATCT
Alcea pall Ruphorbia	TACTUTCATC	GCCCCGTATG	CGGTGTT CCCTCACCCC	TGTGTGGCAG	CTTGACGCTGTT	ACTITIGICG	TGAA	ATACAAAACG	ACTUTUGGCA	ACCONTATCT
Onobrychis	0001 00	GUUUUUATA	001040000	0100	011000	1000001	ITCH CHA	A-CARARCO	ACTUTUDOUA	ACOUNTATO
Teucrium p	CCGCCCCA	CCCCCGTCCC	CGAACC	G <b>77</b> G	GG-ACGAACC	GAAGGCCCTT	CTTA	AACCAAAACA	ACCCTOGGCA	ACGAAATTTT
Thymbra sp	CCTOGCCC	CCCTCCG	CGGCGCGT	-GCTGGGGGA	TCGAGCG	TCCAT	CCCA	TGTCATAACG	ACTCTCGGCA	ACGGATATAT
Verbascum	CCTCTAT	GCCCCGTTCG	CGGTGTGCTC	GGCGGGA	CAGTOCG	TCTCT	TGAA	TGTCATAACG	ACACTTOGCA	ACGGATATCT
Xanthium s	CTATTGC	GCCCCGCTCG	CGGTGTGCGC	-TTTGTACGT	GGCA	TCTTT	C <b>t</b> aa	ACTAATAACG	ACTCTCGGCA	ACGGATATCT
Onopordum_	TCGTGTT	GCCCCGTCCG	CGGTGTGCGC	-ACGGGCCGT	GGCC	TCT	CATTA	ACCATAAACG	ACTCTCGGCA	ACGGATATCT
Umbilicus_	UCT	CTACUGCT-C	GOLOGO	TGCAGGGGCC	TUCAT	TGCTT	CGTT	ATCUTAAACG	ACTUTOUGCG	ATUGAATCTT
Chara sn.	CATCAACTT	GCTGAGCCAT	COUNCIDEUC CGATACCTTC	AACGCAC-OT		CUATCUUA	C	TGCCGAGGC	ACICICUUCA	-TGAGCATCA
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Linum_mucr	CGGCTCTCGC	ATCGATGAAG	AACGTAGCGA	AATGCGATAC	TTGGTGTGAA	TTGCAG-AAT	CCCGTGAACC	ATC		
Haplophyll	TGGGTCTTGT	AACGATAAAG	AACGCTCCCG	GGTGGCCCAT	TCACTGTGCA	TTGCCG-AAT	TOCOTOG-CC	TTCTTTCT		
Reseda auc	CUGCTCTCGC	ATCGATGAAG	AACGTAGCGA	AATGCGATAC	TIGGTGTGAA	TTGCAG-AAT	CCCGTGAATC	ATCGAA		GCGTAAC
Alcea pall	COOCTUTUDC COOCTUTUDC	ATCOATGAAG	AACUTAGOGA	AATOCGATAC	TTOOTOTOAA	TIGCAG-AAT	COORTGAATC	ATCOR		OCGTAAC
Ruphorbia	OGGCTCTCGC	ATCGATGAAG	AACGCAGCGA	AATGCGATAC	TTGGTGTGAA	TTGCAG-GAT	CCCGCGAATC	ATCGAATCGC	TGCGTTCTTC	ATCGCGTAAC
Onobrychis										
Teucrium p	GGGCTCTGGC	ACCOTTAAAA	AAGG <mark>TACC</mark> GA	ATTOCAATAC	TGGGTGTGAA	TTGCAA-AAC	CCCGTGAACC	ACCGTTGC		
Thymbra_sp	CGGCTCTCGC	A <mark>tc</mark> gatcaaa	AACG <mark>T</mark> AACGA	AATGCGATAC	TTGGTGTGAA	TTGCATTAAT	CCCGTGAATC	ATCGAATC		TTTTGAAT
Verbascum	AGGCTCTAGC	ACAGATGAAG	AACG <b>T</b> AGCGA	AATGCGATAC	TTGGTGTGAA	TTGCAG-AAT	CCCGTGAATC	A <mark>tc</mark> gaacg		TCGTAAC
Xanthium s	CGGCTCACGC	ATCGATGAAG	AACGTAGCAA	AATGCGATAC	TTGGTGTGAA	TTGCAG-AAT	CCCGTGAACC	ATC		
Unopordum_	CUGCTCACGC	ATCGATGAAG	AACGTAGCAA	AATGCGATAC	TIGGTGTGAA	TTUCAG-AAT	COULTGAATC	ATCUAGA		GCGTAAC
Glancium f	COURTOTICAC COOPORTONICO	ATCRARCACT ATCRATCAAC	AAUCTACCUA AACGTACCCA	AATGCGATCC AATGCGATAC	TTOCTOTICAA	TIALAA-AAT TTOCAG_AAT	COORTGAATC	ATCIACTC		TOTALC
Chara sp.	CTGCATTCCT	CACGACG-TC	ACCACCTTOG	GTTTCGAAGT	TOOGTOGOGT	CCGTGGTTGT	GOCTTCCCTC	GCTCCCAAC-		TTGGAG
	<u>,</u> 11	1 40	1 40	n , , , , ,	45	1 44	ורג ן	1 .401	1 40	500
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Figure 2: Clustal W multiple sequence alignment of ITS1

and ITS2 regions of the collected species.

The NJ tree is well resolved (bootstrap value >50%)

except for the *Euphorbia Craspedia*, in which the bootstrap value was below 50% (i.e., 48%) (Figure. 3).



Figure 3. Neighbor-Joining tree based on ITS1 and ITS2 regions. The optimal tree with the sum of branch length = 5.67718577. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair.

#### 4. DISCUSSION

The distinct geological structure of the regions (Kharajiany, 2013) made it suitable habitat for plants diversity. Most of the identified species were belong to two clades; Rosids and Atersids. To our knowledge, no occurrence data of; *Onobrychis Ptolemaic, Teucrium oliverianum, Euphorbia Craspedia, Onopordum tauricum, Reseda aucheri subsp. aucheri* were recorded in the Sangaw. On the other hand, the possible reason that the species of some taxa was not able to identify is due to that the species of these taxa are new and not exist in the NCBI database (because the study area is neglected before and no molecular studies on floral composition have been done before). Furthermore, the phylogenetic tree showed the distance among most species is not equal, and this

due that the species belong to different families and have different DNA sequences. The existence of phylogenetic distance between closely related taxa such as *Alcea\_sp* and *Alcea\_pallida* is due to the change in some base pairs (addition or deletion). Such distance is might due to the long-term impacts of abiotic stress in the area such as low precipitation rates and geological structure of the area that cause adaptation and speciation of some species. The phylogenetic distance among coexisting species in the area reveals the existence of phylogenetic diversity among taxa of same family.

Most of the identified species were belong to the rosids clade, and they have different morphology, life span, and reproductive patterns (Folk et al., 2018). Such diversity among members of this clade is considered a critical factor for understanding the evolutionary pattern and ecological dynamics of the angiosperms (Folk, Siniscalchi, & Soltis, 2020). Green algae (Chara sp.) has been found in the downstream of the acid stream. This study showed the importance of using DNA barcoding to identify and classify the taxa and species. Relying only on the classical classification methods (i.e., Morphological characteristics) will not be enough to identify and classify the species. In-plant molecular phylogeny, internal transcript spacer 1 and 2 (ITS1 and ITS2) can identify inter and intraspecific variation (Nilsson et al., 2008; Yao et al., 2010). The newly recorded species in different area of Kurdistan and the finding of this study could support that many new other species could evolve (due to species diversification) in the study area and in Kurdistan. This means that more molecular and morphological taxonomic studies are needed because Kurdistan is a hot spot and need more emphasis.

## **5. CONCLUSION**

The difference in the nucleotide sequence and phylogenetic distance among coexisting species in the area reveals that the identified species are not in the same clade and families and the taxonomic diversity of the area. This study added some new floral taxa found in the area to the flora of Kurdistan. The identified species are new and not recorded before in Sangaw. Therefore, it is important to include molecular methods in species identification process.

#### 6. ACKNOWLEGEMENTS

Many thanks to Dr. Sirwan M. Amin for his assistance with collecting the samples.

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