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Phenotypic characteristics of rhizobial and non-rhizobial isolates recovered from root nodules of chickpea (*Cicer arietinum* L.) grown in Ethiopia

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Fifty endophytic bacteria recovered from surface sterilized root nodules of chickpea (*Cicer arietinum* L.) grown in Ethiopia were authenticated using three chickpea cultivars (Natoli, ICC-4918 and ICC-5003). These isolates were characterized based on morphological and phenotypic traits. The test isolates were divided into two: bacteria nodulating chickpea (27 isolates, Group I] and endophytic non-nodulating bacteria (23 isolates, Group II]. Twenty isolates from Group I were able to re-infect and form symbiosis with two chickpea cultivars, while the remaining seven isolates re-infected only one chickpea cultivar. The result demonstrated that: a number of the tested isolates were able to produce siderophore, showed variations in terms of their resistance to different antibiotics, grew at pH ranging between 5 and 9.5, tolerated salt concentration as high as 2.5% and grew at a temperature as high as 40ºC, indicating the existence of a wide physiological diversity among themselves. Dendrogram construction indicated the existence of four clusters when 78% similarity level was used as cut-off point. Such diversity among the tested isolates showed the presence of diverse rhizobial and non-rhizobial isolates within chickpea nodules. Further investigation aimed at identifying the types of endophytic bacteria, their invading mechanism, and varietal preferences for nodule formation is recommended.

Key words: BNF, Chickpea, Endophytic, *Mesorhizobium,* non-nodulating, rhizobia.

INTRODUCTION

Ethiopia is the largest producer of chickpea in Africa, and the sixth largest producer in the world (Kimurto et al., 2013). The total area of chickpea in Ethiopia has increased from 168,000 – 230,000 ha over the past decade (Central Statistical Agency - CSA, 2014), and the actual yield of chickpea in Ethiopia is below the potential

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yield (Bejiga and Daba, 2006; Keneni et al., 2011a). However, efforts have been made to improve chickpea cultivars with respect to several desirable attributes including yield, tolerance to different diseases/infestation (Ahmed and Ayalew, 2006; Keneni et al., 2011a) and drought (Anbessa and Bejiga, 2002), thus many improved cultivars have been released in Ethiopia (Shiferaw and Teklewold, 2007). However, these efforts alone could not improve the productivity of chickpea in the country. Therefore, the benefits of its biological nitrogen fixation ability with rhizobia should be exploited and integrated together with the above efforts being made so far to enhance the productivity of chickpea in the country and beyond. As a first step to such an approach, we thus isolated and characterized (based on different morphophysiological features) endophytic bacteria (nodulating and non-nodulating) from nodules of chickpea, with the aim to select the best nitrogen fixing rhizobial strains, but also non-nodulating strains having desirable properties for plant growth.

Chickpea forms symbiotic nitrogen-fixation association with *Mesorhizobium* species (Jarvis et al., 1997; Nour et al., 1995). *Mesorhizobium* strains naturally vary in their nitrogen fixing capacity and adaptation to prevailing environmental stresses (L'Taief et al., 2007). Despite high specificity of the legume-*Rhizobium* interaction and the selective nodule environment, the presence of non-nodulating endophtes has been reported; for example, the investigation of *Bacillus* from soybean (Bai et al., 2002), *Agrobacterium* from tropical grass (de Lajudie et al., 1999), 1999), *Klebsiella* from groundnut, clover and bean (Ozawa et al., 2003) and *Pseudomonas* from acacia and soybean (Hoque et al., 2011; Kuklinsky-Sobral et al., 2004), *Shinella* from *Milletia ferruginea* ((Degefu et al., 2013), and non-symbiotic endophytic bacteria from several legumes growing in Ethiopia (Aserse et al., 2013). In most of the aforementioned investigations, it was reported that the non-nodulating endophtic bacteria were shown to have plant growth promoting characteristics (PGP), by mechanisms other than biological nitrogen fixation such as production of Indole-3-Acetic Acids (IAA), sidrophore production and phosphate solubilization. In Ethiopia, except for few studies (Jida and Assefa, 2012; Tena et al., 2017), several undertakings were devoted to breeding of chickpea for adaptation to several stressors including, among others, drought and pest infestations (Ahmed and Ayalew, 2006; Anbessa and Bejiga, 2002; Keneni et al., 2011a; Keneni et al., 2011b). However, in other areas where chickpea is commonly grown, several studies have been conducted on characterization of rhizobia nodulating chickpea and screening of best nitrogen fixing strains for inoculant development (Küçük and Kivanc, 2008; L'Taief et al., 2007; Maâtallah et al., 2002). Nevertheless, except for few studies, similar exhaustive studies are lacking in Ethiopia. This therefore calls for a research aimed at isolation and characterization of endophytic bacteria harboring nodules of chickpea growing in Ethiopia using various morphophysiological features. This study, while having significance on rhizobial biodiversity conservation, will also contribute to sustainable utilization of the nodules' microsymbionts in chickpea production in the country and beyond.

MATERIALS AND METHODS

Chickpea root nodules were collected by the research team of the Debre Zeit Agricultural Research Center during the 2014 cropping season. These nodules were collected from the East, West, and North Shewa zones and West Harerge zone in Ethiopia. Collections were made from randomly selected farmers' fields when most chickpea crops were at flowering stage. The randomly selected fields were on average 15 km apart. These areas have an altitude ranging between 1876 (East Shewa, Ada'a) and 2659 m (North Shewa, Enewari) above sea level. The GPS reading and specific locations are indicated in Table 1. Five healthy plants were uprooted from farmers' field and the nodules were detached from roots aseptically using 70% ethanol and flames for sterilization. Thereafter, the root nodules were put into the vials and filled with silica gel and cotton. The nodules were brought to the Soil Microbiology Laboratory at Hawassa University College of Agriculture for detailed characterization.

Isolation of the chickpea nodule bacteria was made on yeast extract manitol agar (YEMA) medium using the procedure mentioned by Vincent (1970). After a week's incubation, a single colony of chickpea bacteria were isolated and transferred to a new plate. Pure colonies of rhizobia and other endophytic non-rhizobial isolates were transferred onto YEMA slant, for further analysis. Authentication test of the presumptive 50 rhizobial isolates was done in order to distinguish the symbiotic nature of rhizobia from other endophytic isolates. The 3 genotypes used for the authentication test were Natoli, ICC-4918 and ICC-5003. Each of these cultivars were allowed to grow in a Leonard jar and inoculated with 1 ml of isolate broth containing about 10^7 CFU/ml in YEMA broth media. The authentication was replicated 3 times for each chickpea cultivar. The non-inoculated control is taken as a proof of authentication (Maâtallah et al., 2002; Somasegaran and Hoben, 1994). Isolates were evaluated for temperature, pH and salt resistance in YEMA media. The temperature test was made by allowing isolates to grow at temperatures ranging from 5 to 45°C, with an interval of 5°C (8 levels). The pH tolerance capacity of each isolate was tested by growing them on an acidic (pH 4.0, 4.5, 5.0) and alkaline (pH 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0) media of YEMA respectively, and adjusted with 1 N HCl or NaOH solutions (Amarger et al., 1997). The ability of the isolates to grow at different levels of salt concentration were determined by inoculating each isolate on the YEMA media containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5% of NaCl (w/v) (Amarger et al., 1997).

Carbon Source Utilization (CSU) capacity of isolates was determined following the standard procedure described elsewhere (Somasegaran and Hoben, 1994). Nine carbon sources, namely: D-glucose, D- fructose, D-arabinose, D-xylose, maltose, D-sucrose, Trehalose, glycerol and sorbitol were used in the test. Carbohydrates were prepared as 10% (w/v) solution in water. The carbohydrates-free medium, which was essentially similar to YEMA medium, was modified by reducing the yeast extract to 0.05 g/L. Nitrogen Source Utilization (NSU) was determined using different amino acids as mentioned elsewhere (Amarger et al., 1997). Intrinsic Antibiotic Resistance (IAR) was made using the procedure mentioned elsewhere (Somasegaran and Hoben, 1994). Two levels of 4 antibiotics were used for the IAR test. These antibiotics were Ampicillin (5 and 10 $\mu g.ml^{-1}$), chloramphenicol (5

Table 1. Test isolates used, their geographical origins, associated GPS readings and group designation in the present study.

Classification	No.	Zone	District	Latitude, North	Longitude, East	Altitude (m)	Isolates Code
	1	West Shewa	Ambo	08'58"983'	037'01"885'	2428	ICRE01
	2	West Shewa	Dendi	09'01"400'	038'10"763'	2205	ICRE02
	3	West Shewa	Dendi	09'01"400'	038'10"763'	2205	ICRE03
	4	West Shewa	Dendi	09'01"400'	038'10"763'	2205	ICRE04
	5	West Shewa	Dendi	09'01"387'	038'10"854'	2205	ICRE05
	6	West Shewa	Dendi	09'01"387'	038'10"854'	2205	ICRE06
	7	West Shewa	Dendi	09'00"525'	038'17"143'	2155	ICRE07
	8	North Shewa	Enewari	09'52"187'	039'10"697'	2659	ICRE08
	9	West Harerge	Hirna	09'16"692'	041'09"858'	2326	ICRE09
	10	North Shewa	Enewari	09'52"187'	039'10"697'	2659	ICRE10
	11	North Shewa	Enewari	09'52"187'	039'10"697'	2659	ICRE11
	12	Addis Ababa	Akaki	09'53"730'	038'49"283'	2196	ICRE12
	13	Addis Ababa	Akaki	09'53"730'	038'49"283'	2196	ICRE13
Group I	14	Addis Ababa	Akaki	08'47"634'	039'16"628'	2282	ICRE14
	15	Limu	Mojo	08'41"091'	039'10"754'	2012	ICRE15
	16	East Shewa	Ada'a	08'46"365'	039'00"354'	1910	ICRE16
	17	East Shewa	Ada'a	08'41"396'	039'02"655'	1876	ICRE17
	18	West Harerge	Chiro	09'03"815'	040'54"350'	2254	ICRE18
	19	West Harerge	Chiro	09'10"156'	041'02"446'	2212	ICRE19
	20	West Harerge	Chiro	09'10"156'	041'02"446'	2212	ICRE20
	21	West Harerge	Tulo	09'10"680'	041'03"245'	2184	ICRE21
	22	West Harerge	Tulo	09'10"680'	041'03"245'	2184	ICRE22
	23	West Harerge	Tulo	09'10"680'	041'03"245'	2184	ICRE23
	24	West Harerge	Hirna	09'15"189'	041'07"356'	2032	ICRE24
	25	West Harerge	Hirna	09'15"189'	041'07"356'	2032	ICRE25
	26	West Harerge	Kulubi	09'25"391'	041'09"858'	2371	ICRE26
	27	West Harerge	Hirna	09'16"692'	041'09"858'	2326	ICRE27
	1	East Shewa	Ada'a	08°46.411'	038°59.681'	1930	ICNRE01
	2	East Shewa	Ada'a	08°45.991'	038°59.821'	1939	ICNRE02
	3	East Shewa	Gimbichu	08°48.264'	039°00.166'	1977	ICNRE03
	4	East Shewa	Gimbichu	08°48.501'	039°00.203'	1953	ICNRE04
	5	East Shewa	Gimbichu	08°48.780'	039°00.246'	2206	ICNRE05
	6	Finfine Zuria	Legedadi	08°50.998'	039°01.942'	2419	ICNRE06
	7	Finfine Zuria	Menagesha	08°59.610'	039°05.623'	2497	ICNRE07
	8	West Shewa	Ambo	10°35.019'	038°56.067'	2526	ICNRE08
	9	West Shewa	Dendi	10°37.665'	038°54.133'	2472	ICNRE09
	10	West Shewa	Dendi	10°42.420'	038°54.133'	2474	ICNRE10
	11	East Gojam	Bichena	09°58.745'	038°55.188'	2123	ICNRE11
Group II	12	East Gojam	Bichena	09°59.045'	038°55.546'	2313	ICNRE12
	13	East Gojam	Debre work	09°63.010'	038°55.598'	2089	ICNRE13
	14	A/Ababa	Akaki	08°59.817'	038°56.167'	2110	ICNRE14
	15	Finfine Zuria	Gelan	08°58.010'	038°46.073'	2012	ICNRE15
	16	East Shewa	Ada'a	08°40.512'	038°59.310'	1960	ICNRE16
	17	East Shewa	Ada'a	08°39.571'	038°59.435'	1950	ICNRE17
	18	East Shewa	Ada'a	08°33.215'	038°59.214'	1949	ICNRE18
	19	East Shewa	Liben	08°37.012'	038°49.345'	2027	ICNRE19
	20	East Shewa	Ada'a	08°36.358'	038°56.139'	1900	ICNRE20
	21	West Shewa	Ambo	08'59"433'	037'48"022'	1934	ICNRE21
	22	West Shewa	Ambo	08'57"089'	037'55"071'	2225	ICNRE22
	23	West Shewa	Ambo	08'57"089'	037'55"071'	2225	ICNRE23

Table 2. Twenty eight nodule forming rhizobacteria isolates on 3 chickpea seedlings (Natoli, ICC-5003, ICC-4918).

S/N	Chickpea cultivars	Isolates that infected only one variety	Isolates that infected 2 varieties (Natoli, ICC-5003)	Isolates that infected 2 varieties (ICC-5003, ICC-4918)	Isolates that infected 2 varieties (Natoli, ICC-4918)	Isolates that infected all 3 varieties (Natoli, ICC-5003)
1	Natoli	ICRE19, ICRE20, ICRE27	ICRE11, ICRE14, ICRE16, ICRE17, ICRE18, ICRE22, ICRE23	---	ICRE07, ICRE09, ICRE15, ICRE21, ICRE26	ICRE01, ICRE02, ICRE03, ICRE24, ICRE25
2	ICC-5003	ICRE08, ICRE12		ICRE04, ICRE05, ICRE13	---	
3	ICC-4918	ICRE06, ICRE10, ICRE16	---		ICRE07, ICRE09, ICRE15, ICRE21, ICRE26	
Total		8	7	3	5	5

and 10 µg.ml^{-1}), erythromycin (5 and 10 µg.ml^{-1}) and streptomycin (10 and 50 µg.ml^{-1}). The stock solution of each antibiotic was prepared as described before (Zhang et al., 1991). Resistance was determined as resistant (R), intermediate (I) (if any), and susceptible (S) after 5 days of growth.

Intrinsic Heavy Metal Resistance (IHMR) (Al, Cu, Zn, Mn, Pb and Co) was determined on TY agar containing 5.0 g Trypton, 3.0 g yeast extract, 0.87 g $CaCl_2$, 12 g agar and 1000 ml distilled water as described in previous investigation (Zhang et al., 1991). Isolates grown on succinate solution medium (SM) (K_2HPO_4 6 g/L, KH_2PO_4 3 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $(NH_4)_2$ SO_4 1 g/L, succinic acid 4 g/L) pH adjusted to 7.0, were made ready for siderophore production following the procedure described elsewhere (Schwyn and Neilands, 1987). Indole Acetic Acid (IAA) production capacity of each isolate was studied using the log phase culture of each isolate. Exponentially grown cultures of each isolate were incubated separately on nutrient broth medium supplemented with 5 mm L-tryptophan (100 µg/ml) for 72 h (Bric et al., 1991). Mixtures were incubated for 25 min and were observed for the development of pink color. Amylase production test was made on starch agar plates as described before (Cappuccino and Sherman, 2008). The isolates were streaked on starch agar plates and incubated at 28°C. Amylase production was detected by flooding the plates with iodine solution (Hols et al., 1994). Cellulase production test was made on carboxyl methyl cellulose agar, as mentioned before (Bhatt and Vyas, 2014) by flooding with an aqueous solution of Congo red (1% w/v). Cluster analysis of phenotypic variables was worked out using a similarity coefficient, by the un-weighed pair group method with the average (UPGMA) clustering, using NTSYspc21 version software.

RESULTS AND DISCUSSION

Isolation of root nodule bacteria

A total of fifty endophytic bacteria from nodules of chickpea growing in diverse localities in Ethiopia (Table 1) were used for this study of which twenty-seven (54%) isolates were able to re-infect roots of chickpea cultivars. Out of the 27 isolates, 20 (74%) were able to re-infect a minimum of 2 chickpea cultivars seedlings, while 7 (25.9%) isolates re-infected only one chickpea cultivar. The 27 isolates capable of re-infecting chickpea seedlings were the true rhizobial isolates and were designated as Group I. However, the isolates had their own cultivar preference. The remaining 23 (46%) isolates that were not able to re-infect seedlings to form nodules with any of the tested chickpea cultivars were designated as Group II (endophytic non-rhizobial bacteria) (Table 1). Both nodulating isolates and endophytic non-nodulating bacterial isolates were characterized based on their physiological and biochemical properties. The investigation of both nodulating and non-nodulating endophtic bacteria from nodules of chickpea in the present study is becoming a common place phenomena, since several studies have also reported similar results from nodules of diverse legume species (Aserse et al., 2013; Degefu et al., 2013; Gurtler et al., 1991; Liu et al., 2005; Mahdhi et al., 2008). The reason why nodulating members were present in nodules of chickpea is obvious, as they provide the host plant with fixed nitrogen through biological nitrogen fixation process. With respect to the non-nodulating endophytes, they could also promote plant growth by mechanisms other than biological nitrogen fixation as evidenced in several in vitro investigations (Alikhani and Yakhchali, 2010; Alikhani et al., 2006; Aserse et al., 2013; Mahdhi et al., 2008). However, the current available reports from different findings are inconclusive, thus the comprehensive mechanisms as to how they offer growth to host plant remains to be seen.

As indicated in Table 2, only 5 isolates were capable of re-infecting and forming nodules on all 3 chickpea cultivars. Two alternate cultivars of Natoli and ICC-5003, ICC-5003 and ICC-4918, and Natoli and ICC-4918, formed root nodules in symbiosis with 7, 3 and 5 isolates, respectively (Table 2). The 27 isolates that exhibited the ability to re-infect chickpea seedlings had their own varietal preference. It has been well demonstrated that nodule formation and biological nitrogen fixation is a function of the genotypes of legumes and rhizobia (Giller et al., 2013), which corroborates our present findings. The 3 chickpea cultivars Natoli, ICC-5003, and ICC-4918, were capable of forming root nodules in symbiosis with

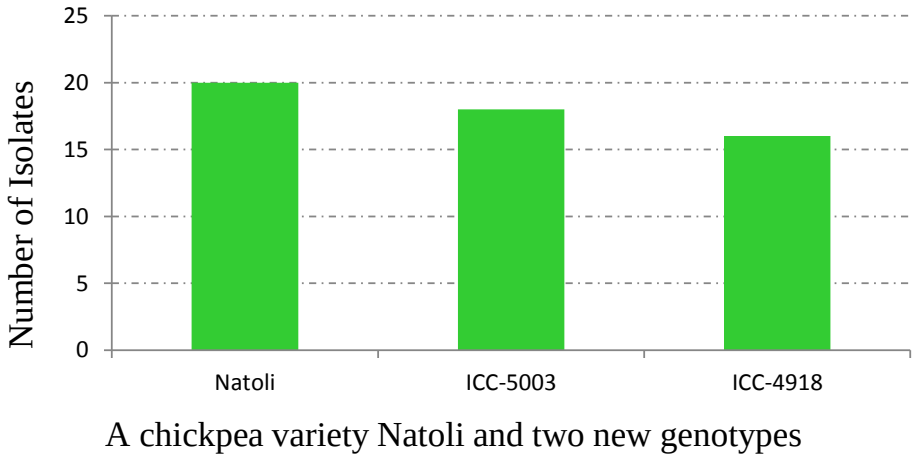

Figure 1. Number of isolates capable of re-infecting and forming nodule on different chickpea cultivars.

20, 17 and 16 isolates, respectively (Figure 1).

Bacterial growth and morphological characteristics

On the basis of their generation times, 18% of the tested isolates belong to Group I, and 6% of test isolates circumscribed within Group II were fast growers with generation time ranging between 1.8 and 2.8 h. 74% of the test isolates (comprising the two groups) were found to be slow growers (with generation time exceeding 3 h and reaching as high as 9 h). Isolate ICRE13 was an extra-slow grower with a generation time that exceeded 9 h. This is consistent with earlier studies of similar nature, reporting that chickpea is being nodulated with rhizobia comprising fast, slow and extra-slow growers. For example, chickpea rhizobia have previously been shown to possess both fast and slow growing strains (Chakrabarti et al., 1986). It was reported that moderately slow growing rhizobia *Mesorhizobium mediteraneum* is specific but also the natural symbionts of chickpea (Nour et al., 1995). Furthermore, in another study of similar nature, 22% of the isolates from chickpea were fast growers, 32% slow growers and 46% extra-slow growing bacteria with a generation time of more than 9 h (Maâtallah et al., 2002). With the exception of few test isolates including ICRE06, ICRE08, ICRE09, ICRE13, ICRE16 and ICRE17 from Group I, and ICNRE01, ICNRE09, ICNRE16, ICNRE17, ICNRE19 and ICNRE20 from Group II, the majority of the tested isolates did not absorb Congo red. This is a distinctive character of rhizobia as already reported elsewhere (Somasegaran and Hoben, 1994) (Table 3).

All strains from both groups were found to have circular colonies with regular borders, flat in elevation, creamy in color, showing intermediate to high production of mucus. 70% of the test isolates (Groups I and II) were characterized with large mucoid texture, whereas, 24% of the isolates appeared as large watery colonies. The colony diameter of all the test isolates within the two defined groups ranged between1.8 and 9.8 mm except for isolates ICRE07 and ICRE22, which showed a colony diameter less than 1 µm. 38% of the tested isolates included within the two groups were shown to produce gum when plated on YEMA media. The majority of the tested isolates produced yellow color on BTB medium indicating that they are acid producers (Vincent, 1970), except isolates ICRE18, ICRE22, ICRE25 and ICRE26 that produced blue color on BTB medium (Table 3). Most of the tested isolates (representing Groups I and II) did not grow on BCP medium except 3 isolates (ICRE09, ICRE17 and ICRE20) from Group I, and 2 isolates (ICNRE04 and ICNRE14) from Group II (Table 3). In an earlier study, it has been well documented that rhizobia were unable to grow on BCP media (Somasegaran and Hoben, 1994). However, this research finding contradicts previous reports of Somasegaran and Hoben (1994). In agreement with this study findings, other studies reported that rhizobial could also grow well on BCP medium indicating the capability of the isolates to use glucose as sole carbon source (Küçük et al., 2006).

Physiological and biochemical tests

pH tolerance

All the isolates (both Groups I and II) tolerated a pH of 8, whereas 86% of both groups grew at a pH of 5.5 (Table S1). This is consistent with the findings of other studies on rhizobia from chickpea growing in Ethiopia (Jida and Assefa, 2012), who reported that all tested isolates from nodules of chickpea grew well in moderately acidic pH (5.5) to neutral pH and slightly alkaline pH (8.0). As indicated in Figure 2, 51% of isolates were tolerant to a lower pH of 4. Very few isolates (5%) were able to resist

Table 3. Isolate code, Congo red absorption on CR-YEMA media (described as either S=slightly absorbed the CR-YEMA medium or No= did not absorb CR-Medium), colony color on BTB (described as Y=for yellow, B=for blue) and growth on BCP (described as '+'=for growth, '-'=for no growth), gum production ('+'=produced gum, '-'=did not produce gum) and mean generation time (in hours) of the tested isolates.

#	Isolate code	CR-YEMA	BTB	BCP	Colony Diameter (mm)	Gum Production	Mean Generation Time
1	ICNRE01	S	Y	-	5.1	-	3.8
2	ICNRE02	No	Y	-	1	-	6.8
3	ICNRE03	No	Y	-	0.7	-	3.2
4	ICNRE04	S	Y	+	3.3	-	2.2
5	ICNRE05	No	Y	-	3.9	+	3.3
6	ICNRE06	No	Y	-	1.6	+	2.1
7	ICNRE07	No	Y	-	1.4	-	3.5
8	ICNRE08	No	Y	-	1.9	-	6.2
9	ICNRE09	S	Y	-	1.2	+	7.1
10	ICNRE10	No	Y	-	3.5	+	3.7
11	ICNRE11	No	Y	-	4.9	+	5.6
12	ICNRE12	No	Y	-	5.1	+	4
13	ICNRE13	No	Y	-	5.1	+	3.7
14	ICNRE14	No	Y	+	5.5	-	2.6
15	ICNRE15	No	Y	-	3.1	-	3
16	ICNRE16	S	Y	-	3	-	1.9
17	ICNRE17	S	Y	-	3.6	+	5.5
18	ICNRE18	No	Y	-	1.4	+	3.2
19	ICNRE19	S	Y	-	4.5	-	5.2
20	ICNRE20	S	Y	-	5.8	-	2.1
21	ICNRE21	No	Y	-	5.6	-	4.3
22	ICNRE22	No	Y	-	2.3	-	2.8
23	ICRE01	No	Y	-	3	+	1.8
24	ICRE02	No	Y	-	3	-	2.6
25	ICRE03	No	Y	-	1.7	-	3.8
26	ICRE04	No	Y	-	1.7	-	4
27	ICRE05	No	Y	-	2	-	5.1
28	ICRE06	S	Y	-	2.9	+	1.9
29	ICRE07	No	Y	-	0.7	-	3.2
30	ICRE08	Yes	Y	-	3.4	-	4.5
31	ICRE09	S	Y	+	3.3	-	2.2
32	ICRE10	No	Y	-	1.4	-	4.8
33	ICRE11	No	Y	+	1.5	-	4.9
34	ICRE12	No	Y	-	4.9	-	4.7
35	ICRE13	No	Y	-	2.3	-	4.6
36	ICRE14	No	Y	-	2.1	+	4.8
37	ICRE15	S	Y	-	4.8	+	9.8
38	ICRE16	S	Y	-	1.5	+	3.6
39	ICRE17	S	Y	-	2.3	+	1.8
40	ICRE18	No	B	-	3.5	-	5.1
41	ICRE19	No	Y	-	1	-	3.3
42	ICRE20	No	Y	+	4.1	-	4.9
43	ICRE21	No	Y	-	1.5	+	2.5
44	ICRE22	No	B	-	0.9	-	2.3
45	ICRE23	No	Y	+	5	-	6.2
46	ICRE24	No	Y	-	2.6	-	3.9
47	ICRE25	No	B	-	2	-	6.4
48	ICRE26	No	B	-	3.5	-	2.8
49	ICRE27	No	Y	-	5.3	+	3.1
50	ICRE28	No	Y	-	2	+	3.7

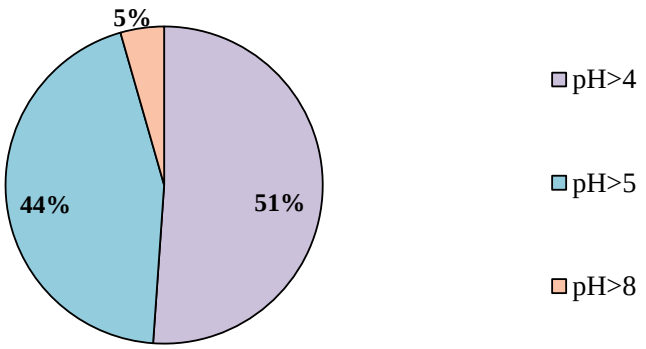

Figure 2. Proportion of chickpea nodule endophytes tolerance to different pH values.

a pH of 8 to 10. 30% of Groups I and II isolates grew at pH value of 4, whereas 72% of Groups I and II test isolates were able to tolerate a pH of 10.5. The pH can be a major limiting factor for growth of a number of microorganisms in the soil (Brockwell et al., 1991). Considering pH as one of the parameters influencing the survival and growth of microorganisms in the soils, the tested strains with broad range of pH preference could have a selective advantage over those requiring narrow pH ranges. Nevertheless, the survival and growth of those strains remains to be seen under field conditions with contrasting pH values before developing them into inoculant.

Salt tolerance

All the tested isolates (Groups I and II) were able to grow on YEMA containing 0.5% of NaCl, but showed steady decrease in growth as the concentration of salt kept increasing. As indicated in Table S1, the test isolates showed variations to high NaCl concentration. For example, 76% of Group I isolates grew at a salt concentration of 1% NaCl. These results are in agreement with other findings who reported 75% of the tested rhizobial inoculants grew well with 1% NaCl (Jida and Assefa, 2012). All isolates were tolerant to the lowest level of NaCl concentration. Of the tested isolates, only ICRE25 and ICRE28 from Group I and ICNRE11 and ICNRE22 from Group II were able to tolerate NaCl concentration of 4%. Only one isolate from Group I (that is, ICRE08) was found to grow at a NaCl concentration of 5%. This osmo-tolerant isolate may be considered as a potential candidates for inoculant development for localities where salinity is a problem, as salinity is reported to decrease the efficiency of rhizobium legume symbiosis (Bouhmouch et al., 2001; Hashem et al., 1998).

Temperature tolerance

High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops, since temperature affects almost all stages of legume-rhizobia symbiosis (Hungria and Vargas, 2000; Zahran, 1999). High root temperatures strongly affect bacterial infection and N_2 fixation in several legume species. For most rhizobia, the optimum temperature range for growth in culture is 28 to 31°C, and many are unable to grow at 37°C (Graham, 1992). Tolerance of the isolates to different temperature levels were tested and are presented in Table S1. 40% of the test isolates tolerated temperatures up to 40°C. Fifteen isolates were tolerant to the lower level of temperature (5°C). In the present study, all Groups I and II isolates grew at a temperature of 20-30°C, whereas 32% of isolates (10 isolates from Group I and 6 isolates from Group II) grew at a temperature of 5°C. The results of this study are consistent with previous findings (Berrada et al., 2012; Zahran, 1999), which showed that rhizobia are mesophiles, and can grow at temperature values ranging between 10 and 37°C. 42% of isolates (12 from Group I and 9 from Group II) were able to grow at a temperature of 40°C. Similarly, growth was reported for both *M. ciceri* and *M. mediterraneum* type strains, which are the natural symbiont of chickpea, at a temperature of 40ºC (Nour et al., 1995). Therefore, it can be suggested that the thermophilic isolates investigated in this study might be the presumptive candidates for inoculant development in areas under extreme temperatures. However, the in vitro results reported in the present study cannot be conclusive, thus those strains should further be screened with the aim to investigate their performance under field conditions.

Carbon source utilization

66% of the chickpea isolates were able to utilize above 80% of carbon sources tested (Table S1). 22% of Group I isolates were able to utilize all carbon sources, while 30% of Group II isolates were able to utilize all carbon sources. 66% of isolates (comprising the two groups) were able to utilize fructose. These are in line with the findings of others from Ethiopian casess who reported that isolates were capable of utilizing fructose as their sole carbon source (Keneni et al., 2010). In another study, it was reported that the slow growing rhizobia grow best on pentose as their carbon source and fast growers usually grow best on glucose or sucrose as their sole carbon source (Somasegaran and Hoben, 1994). However, in the present study, preference for a carbon source seems not to be influenced by being fast or slow growing rhizobia. Concordant with this, different carbon sources could not discriminate the fast and slow growing isolates (Hameed et al., 2004).

Nitrogen source utilization

Similar to the CSU, 66% of the chickpea isolates were

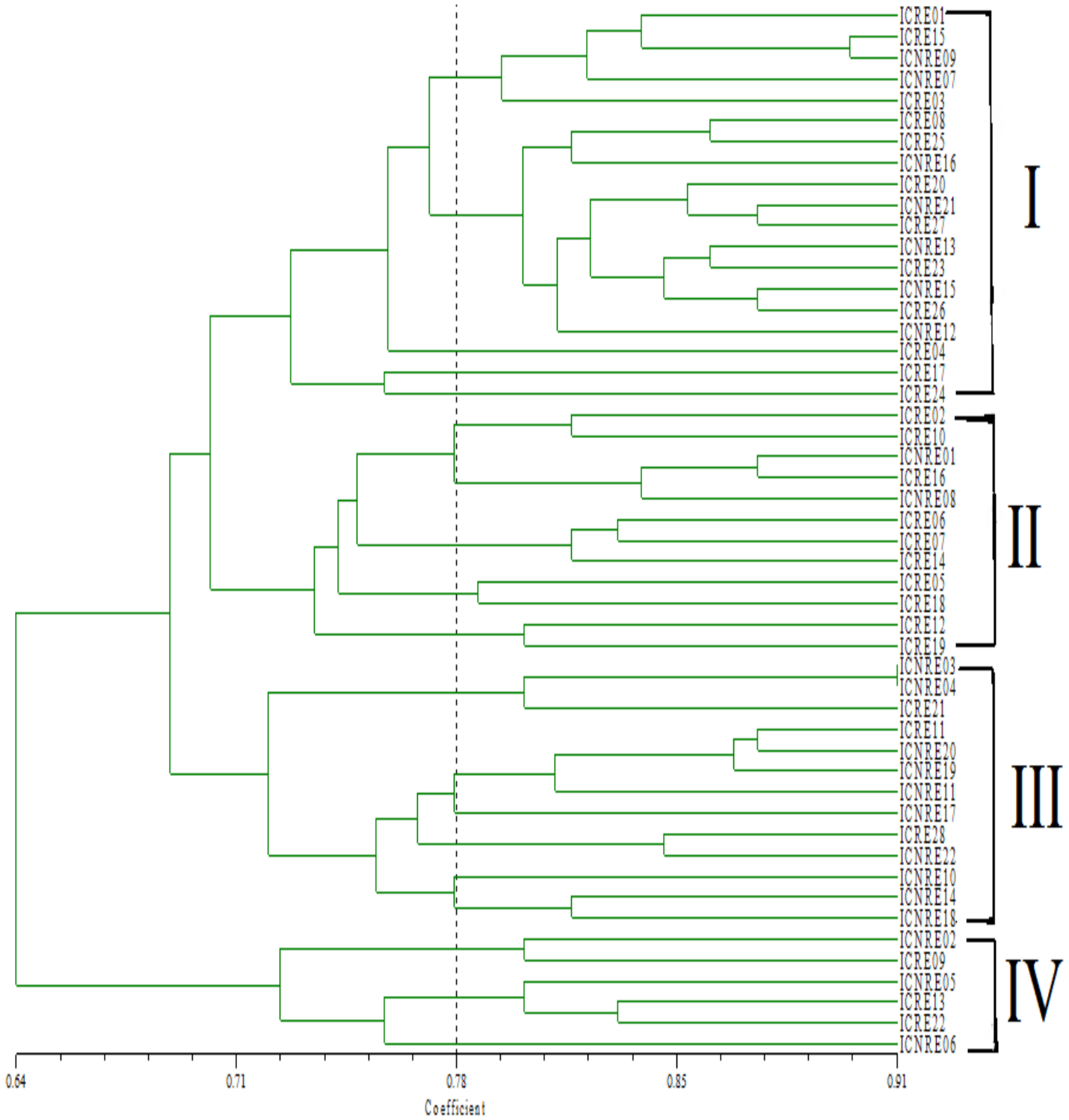

Figure 3. Dendrogram highlighting the phenotypic similarities among non-nodulating and rhizobia nodulating chickpea growing in Ethiopia.

able to utilize above 80% of nitrogen sources (Table S1). The isolates were capable of utilizing amino acids as their sole nitrogen source. All the isolates (comprising the two groups) catabolized L-proline. Two isolates, ICNRE13 and ICNRE15, catabolized all tested amino acids. 96% of Group I and Group II were able to utilize L-Asparagine. Contrary to the findings of this study, it was reported that none of the rhizobia strains utilized the amino acid L-

asparagine (Zhang et al., 1991). In the present study, chickpea rhizobia exhibited diversity in utilizing different N sources. Hence, the finding of this study agreed with other reports of others who reported that many amino acids were found to serve as sole nitrogen sources to rhizobia isolates (Küçük et al., 2006). The ability of the tested isolates to utilize a wide range of amino acids as sole nitrogen source could be considered as a desirable trait for bacterial survival and growth in areas where nitrogen source is a limiting factor.

Intrinsic antibiotic resistance (IAR)

All endophytic bacteria were assessed for their IAR pattern against two doses of Ampicillin, Chloramphenicol, Erythromycin, and Streptomycin. As indicated in Table S1, all the tested isolates (comprising the two groups) were resistant to Erythromycin at a concentration of 5 µg/ml. Only isolates ICNRE22 (Group II) and ICRE03 (Group I) were resistant to 10 µg/ml Neomycin and 10 µg/ml Kanamycin, respectively. Streptomycin, Neomycin and Kanamycin were found to be the most potent antibiotics that allowed the growth of few isolates. The pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia (Somasegaran and Hoben, 1994). The isolates in this study showed variation in their resistance to the tested antibiotics. Similarly, great variations among chickpea rhizobia with respect to IAR pattern were detected in other studies (Küçük and Kivanc, 2008; Maâtallah et al., 2002). It has been reported that fast-growing strains are more sensitive to antibiotics than slow-growing rhizobia (Maâtallah et al., 2002). Conversely, in this study, the tested isolates comprising of fast, intermediate and slow growing members, showed a wide range of behavior with regard to antibiotics. It can be suggested that, the tested isolates with high resistance to different antibiotics could be selected as a candidate for inoculant development for localities where antibiotics production by different soils dwelling microorganisms are a common phenomenon.

Indole acetic acid (IAA)

Results for Indole Acetic Acid (IAA) production suggest only 33% of these isolates were able to produce it. The Indole Acetic Acid produced by rhizobia accelerates elongation of root hairs and lateral roots, thus improving crop growth (Okon and Kapulnik, 1986). Several researchers demonstrated that rhizobia are capable of producing IAA (Etesami et al., 2009; Sridevi and Mallaiah, 2007). 30% of chickpea isolates in this study produced IAA (Table S1). In another study of similar nature, it was reported that 61.6% of the tested isolates produced IAA (Trivedi et al., 2011), while 12 isolates (out of the 16 tested isolates) were shown to produce IAA (Rani et al., 2011). Endophytic bacteria (nodulating and non-nodulating ones) within the root nodules of legumes were reported to have several desirable attributes, for example, the ability to promote plant growth (Alikhani and Yakhchali, 2010) through the synthesis of phytohormones such as IAA.

Intrinsic heavy metal resistance

In the present study, 74% of tested isolates (comprising the two groups) were resistant to $MnSO_4$, whereas 24% of them were resistant to Pb (COOH) (Table S1). Fifty and 26% of isolates (comprising the two groups) were resistant to Cu and Zn, respectively. The significant importance of resistance of heavy metals to isolates indicates their ability to survive in soil contaminated with heavy metals as described elsewhere (Alikhani and Yakhchali, 2010).

Enzymes production

The isolates were tested for amylase, catalase and Cellulase production. 92% of the isolates (26 from Group I and 20 from Group II) were able to produce amylase. 78% of Groups I and II isolates were able to produce catalase. 66% of the isolates (20 from Group I and 13 from Group II) were able to produce Cellulase (Table 3). These findings are in line with another study of similar nature (Bhatt and Vyas, 2014), who reported production of amylase, catalase and cellulose by chickpea isolates. Furthermore, it was also reported that 64% isolates from chickpea were able to produce cellulase on carboxymethylcellulose (CMC) medium (Bhagat et al., 2014).

Siderophore production

Siderophores are known to bind to the available form of iron (Fe^{3+}) in the chickpea rhizosphere thus making it unavailable to the phytopathogens and consequently protecting plant health (Wani and Khan, 2013). 90% of the tested isolates (25 from Group I and 20 from Group II) exhibited positive results for siderophore production test (Table S1), which is similar to the work of others (Bhagat et al., 2014), who reported 88% of chickpea isolates were able to produce siderophores. Additionally, in other studies, it was reported that Mesorhizobium species recovered from nodules of chickpea produced siderophore (Küçük and Kivanc, 2008; Raychaudhuri et al., 2005). The fact that the test strains were able to produce siderophore was an indication that those strains may protect their host by outcompeting pathogens, thus enhancing the growth of the host plant.

Cluster analysis

The result of the cluster analysis performed on the 50 test isolates recovered from root nodules of chickpea growing in diverse chickpea growing localities in Ethiopia for 74 phenotypic traits, is shown in Figure 3. All the isolates examined were grouped into 4 (designated as I-IV) clusters, when 78% similarity level was used as a cutoff point.

Isolates in cluster I were characterized by surviving pH values ranging between 4 and 10.5. None of them in this cluster survive salt concentration greater than 3.5 except one isolate (ICNRE13) belonging to Group II. In this cluster, seven isolates (3 from Group I and 4 from Group II) were capable of surviving higher temperatures of 40°C. All Groups I and II isolates in cluster I were sensitive to Neomycin and Kanamycin. Ten of Group I isolates in cluster I showed phosphate solubilization. All Groups I and II isolates in cluster I were able to produce siderophore. None of Group I isolates in cluster II were capable of growing at pH 4 whereas, all of them grew at pH 5 - 9.5 except ICRE12 which failed to survive at pH 9.5. All Groups I and II isolates in cluster II were capable of surviving salt concentrations up to 2%. Only isolate ICRE16 of cluster II was able to grow at a temperature of 45°C. Almost all of Groups I and II isolates in cluster II were highly sensitive to Neomycin, Chloramphenicol, Streptomycin and Kanamycin. Isolate ICRE19 solubilized phosphate and isolate ICRE07 produced IAA. In addition, all Groups I and II isolates within cluster II were able to produce catalase and cellulase, while all isolates except ICRE08 and ICRE14 were able to produce Amylase in this cluster. In cluster III, all Groups I and II isolates were resistant to 2% salt concentration, whereas 76.4% of isolates (comprising the two groups) were able to grow at 3% salt concentration. All isolates except ICNRE11, ICNRE15 and ICNRE20 were able to grow at a temperature between 15 and 35°C. None of the isolates in this cluster were able to grow at 45°C.

The fourth cluster contained 5 isolates. The growth of isolates in cluster IV was limited to pH values 5.5 and 8. None of the isolates (comprising the two groups) in cluster IV were able to survive at pH values less than 5.5 and greater than 8, except isolate ICRE13 that was able to grow at pH value 8.5. All Groups I and II isolates in cluster IV were unable to tolerate salt concentration of more than 1%.

Isolates ICNRE05 and ICRE13 were unable to grow at salt concentration of 1%, but they were able to survive temperatures between 10 and 40°C in cluster IV. With regard to intrinsic antibiotic, all Groups I and II isolates in cluster IV were highly sensitive to Neomycin, Kanamycin, Streptomycin and Chloramphenicol at all tested concentrations. All Group I isolates in cluster IV were capable of resisting $MnSO_4$. Isolates ICRE13 and ICRE22, were able to produce siderophore and solubilize phosphate.

Conclusion

This study revealed enormous diversity among the tested isolates. Rhizobial and non-nodulating endophytic bacteria harboring the chickpea root nodule collections were almost equal in proportion. Nodulating group, in this study showed desirable physiological characteristics that could be further verified under field condition to develop inocula for chickpea. Although the non-nodulating endophytic bacteria (Group II in the present study) harboring root nodules of chickpea could not infect the chickpea to produce nodules, they showed very positive physiological characteristics that could accelerate seedling emergence, promoting plant establishment under adverse conditions. However, results from the present study are inconclusive, thus detailed molecular mechanisms revealing their effects and functions in plant growth need to be comprehensively defined. Taken together, the existence of enormous diversity among the test isolates reveal the presence of diverse rhizobial and non-rhizobial isolates within the nodules of chickpea in the study sites. However, more exploration and characterization is required to unearth unidentified but existing endophytic bacteria resident within the nodules of chickpea growing in Ethiopia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Physiological and biochemical characterization of endophytic isolates.

Isolates	pH tolerance	Salt (%) tolerance	Temp. (^{o}c) tolerance	C- Sources Utilized (%)	N-sources Utilized (%)	Antibiotics resistance		Intrinsic Heavy Metals Resistance	Enzymes production			IAA production	PO_4 solublization	Siderophore production
						5%	10%		Amylase	Catalase	Cellulase			
ICRE01	4.5-11	0.5-2	10-30	91	91	Ne, Ch, Er,	Er, St,	Al, Cu	+	-	+	+	+	+
ICRE02	5.5-10.5	0.5-2	10-30	73	82	Er	Er	Mn, Al, Zn	+	+	+	-	-	+
ICRE03	4-10.5	0.5-3	10-40	91	91	Er, Ka	Er, Ka	Mn, Co, Cu	+	-	+	-	+	+
ICRE04	4-11	0.5-2	10-35	73	73	Ch, Er, Ka	Ch, Er	Pb, Mn, Al, Co	+	+	+	+	-	+
ICRE05	5-10.5	0.5	20-40	54	82	Er	Er	Pb, Mn, Al,	+	+	+	-	-	+
ICRE06	4.5-11	0.5	15-30	73	63	Ch, Er	Ch, Er	Mn	+	+	+	-	-	+
ICRE07	4.5-10.5	0.5	15-35	72	54	Er	Er	Mn, Al, Co	+	+	+	+	-	+
ICRE08	4-10.5	0.5-2.5	10-40	91	73	Ne, Ch, Er	Er, Str	Mn	+	+	+	+	-	+
ICNRE01	4.5-10.5	0.5-2	10-40	91	91	Er	Er, Ka	Pb, Mn, Al	-	+	+	-	-	-
ICNRE02	5-5.5	0.5-1	10-30	54	73	Er	Er	Mn	+	+	-	-	-	+
ICRE09	8	0.5-1	20-30	63	73	Er	Er	Mn, Al, ,Cu	+	-	+	-	-	+
ICNRE03	5.5-10	0.5-2	5-40	73	91	Er	-	Al, Cu	+	+	-	+	+	+
ICNRE04	5.5-10	0.5-2	5-40	82	82	Er	-	Mn, Co, Cu	+	-	-	+	+	+
ICRE10	5-10	0.5-2	10-30	91	82	Er	Er	Mn, Al, Co, Zn	+	+	+	-	-	+
ICRE11	8-10	0.5-4	5-40	91	73	Er	Er	Mn, Al, Cu, Zn	+	+	+	-	-	+
ICNRE05	5.5-8	0.5	10-40	82	91	Er	-	Pb, Mn, ,Cu,	-	+	-	+	-	-
ICRE12	5-9	0.5-2	15-35	73	82	Er	Er	Pb, Zn, Co	+	+	+	-	-	+
ICNRE06	5.5-9	0.5-2.5	5-35	82	91	Er	-	Cu, Al, Co	+	+	+	-	-	+
ICRE13	8-8.5	0.5	10-40	100	91	Er	-	Pb, Mn, Co,	+	-	+	-	+	+
ICRE14	5-10.5	0.5	10-30	73	82	Er	-	Mn, Co	-	+	+	-	-	-
ICRE15	4-11	0.5-2	35-40	91	91	Er, Ka	Er	Mn, Al, Co	+	-	-	+	+	+
ICNRE07	4-11	0.5-2.5	15-35	100	91	Er	Er	Mn, Zn	+	+	+	-	+	+
ICNRE08	5-11	0.5	10-35	100	73	Er	Er	Pb, Mn, Al	-	-	+	-	+	-
ICNRE09	4-11	0.5-2	10-30	100	82	Er	Er	Mn, Co	+	-	+	+	-	+
ICRE16	5-10.5	0.5	10-45	100	91	Er	Er	Mn, Al	+	+	+	-	-	+
ICRE17	4-10.5	0.5	15-45	91	63	Er	Er	Mn,Al	+	+	-	+	+	+
ICRE18	5-9.5	0.5	15-35	63	63	Er	Er	Pb, Al, Cu, Zn	+	+	+	-	-	+
ICRE19	5-10.5	0.5-1.5	15-35	100	63	Er	Er, Str.	Pb, Al, Co, Zn	+	+	+	-	+	+
ICRE20	4-10.5	0.5-2	5-40	91	82	Ne, Ch, Er	Er,	Mn, Cu, Al,	+	+	+	+	-	+
ICNRE10	5.5-10.5	0.5-4	5-30	36	73	Er.	Er.	Mn, Cu Co,	+	+	+	-	-	+
ICRE21	5-10.5	0.5-2	5-40	63	82	Ch, Er.	Er.	Mn	+	+	-	+	-	+
ICNRE11	4-10.5	0.5-5	5-40	82	91	Er,	Er,	Pb, Cu, Co	+	+	-	+	-	+
ICRE22	5.5-8	0.5-1	5-30	100	91	Er,	Er	Pb,Mn,cu,co,	+	+	-	-	+	+
ICNRE12	5-10.5	0.5-1	5-30	91	82	Ne, Er,	Er, Str.	Mn,cu Al,Zn	+	+	-	-	+	+
ICNRE13	5-10.5	0.5-4	5-35	91	100	Ch, Er,	Ch Er,	Al, Mn,	+	+	-	-	+	+
ICNRE14	8-11	0.5-3.5	10-35	73	91	Er	Er	Cu, Al,	+	+	+	-	+	+
ICNRE15	4.5-11	0.5-1.5	5-40	100	100	Ch, Er,	Ch, Er,	Mn, Cu, Al,	+	-	+	-	+	+
ICRE23	4-11	0.5-3	1o-30	91	91	Ch, Er,	Ch, Er	Cu, Al	+	+	+	-	+	+
ICRE24	4-11	0.5	5-30	100	54	Ch,Er	Ch,Er	Mn, Al, Co	+	-	+	-	+	+
ICRE25	4-11	0.5-2	10-35	91	63	Ne Ch, Er	Ch, Er	Cu, Al,	+	+	-	-	-	+
ICNRE16	4-11	0.5-3.5	10-45	91	82	Ne, Ch, Er	Ch, Er	Pb, Mn, Cu,	+	+	-	-	+	+
ICNRE17	4.5-10	0.5-4	10-35	73	82	Er	Er	Al, Zn, Co	+	+	-	+	+	+
ICNRE18	5-8.5	0.5	15-35	54	82	Er	Er	Pb, Mn, Zn,	+	+	+	-	+	+
ICNRE19	5.5-8.5	0.5-4	5-30	100	91	Er	Er	Mn, Al, Co	+	+	-	-	+	+
ICNRE20	5.5-10.5	0.5-4.5	5-40	91	82	Er	Er	Co	+	+	+	-	+	+
ICNRE21	4.5-10	0.5-2	10-35	100	91	Ne, Ch, Er	Ne, Er, C	Cu	+	+	+	-	-	+
ICRE26	4.5-10.5	0.5-2	10-40	91	91	Ne, Ch, Er	Er, Ch	Mn, Cu, Al,	+	+	-	-	+	+
ICRE27	4.5-10.5	0.5.2	10-30	100	91	Ne Ch, Er	Ch, Er	Pb, Cu, Zn,	+	+	-	+	-	+
ICRE28	4-10.5	0.5.4.5	5-40	91	63	Ch, Er	Ch, Er	Mn, Al	+	-	+	-	-	+
ICNRE22	4-10.5	0.5-4.5	5-35	100	82	Ne, Er	Ne, Er	Mn, Al	+	+	+	-	-	+