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ISOLATION OF POTENTIAL PHOTOSYNTHETIC N₂-FIXING MICROBES FROM TOPSOIL OF NATIVE GRASSLANDS IN SOUTH DAKOTA

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♯Shengni Tian and Nanfang Wang are co-first authors who made equal contributions.

ABSTRACT

Nitrogen fertilizer is one of the most limiting factors and costly inputs in agriculture production. Current fossil fuel-dependent ammonia production is both energy intensive and environmentally damaging. An economically practical and environmentally friendly solution for the production of ammonia is urgently needed. Solar-powered N₂-fixing cyanobacteria provide a unique opportunity and promise for applications in agriculture compared to all other N₂-fixing bacteria that cannot use solar energy. Isolation of nitrogen-fixing microbes from the topsoil of native grasslands may have the potential to use them in crop fields as living ammonia factories. This may be a mechanism to free farmers from heavy reliance on fossil fuels-dependent chemical nitrogen fertilizers and to improve soil health for sustainable agriculture. To screen for solar-powered N₂-fixing cyanobacteria in topsoil of native grasslands in South Dakota, we collected 144 topsoil samples from several native grasslands. Six photosynthetic microbial strains were isolated that are capable of growing well autotrophically in a nitrogen-free medium, suggesting that these six microbial strains have the ability to fix N₂. They were assigned the names: Xu15, Xu81, Xu86, Xu111, Xu141, and WW3. Based on cell morphology and its 18S rRNA gene sequence that we obtained, strain Xu15 was reassigned as *Chloroidium saccharophilum* Xu15, a common terrestrial coccoid green alga.

An acetylene reduction assay detected substantial ethylene production, suggesting nitrogenase activity occurrences in cultures Xu81 and Xu15. The other four are in the process of purification for testing their nitrogenase activity. Xu81, Xu111 and Xu141 are probably unicellular microalga, while WW3 and Xu86 are

likely filamentous cyanobacteria. Future research will focus on developing these validated N₂-fixing microbes as *in situ* living ammonia factories in crop fields.

Keywords

Sustainable agriculture, Nitrogen-fixation, Cyanobacteria, Microalgae, Soil health, Bio-solar ammonia factory

INTRODUCTION

Today's unsustainable supplies and volatile prices of fossil fuels have dramatically increased the price of ammonia fertilizer produced by the Haber-Bosch process, which is energy intensive and also contributes to greenhouse gas emissions. According to the report from the Food and Agriculture Organization of the United Nations (FAO), more than 188.31 million tons of ammonia will be needed annually to support the world's food production by 2020, and it is expected to grow annually on average by 1.5 percent (FAO 2017). The economic and environmental costs of the heavy use of fossil fuel-dependent, chemically synthesized ammonia fertilizers in modern agriculture have become pressing problems. Therefore, economical, environment-friendly sustainable production of ammonia must be urgently sought. Solar energy-driven nitrogen fixation by some cyanobacteria offers such a unique opportunity. In addition to reduced costs, cyanobacteria-based nitrogen fixation using solar energy and consuming CO₂ may help alleviate the two global problems: fossil fuel depletion and environment degradation due to elevated CO₂ emissions.

Biological nitrogen fixation refers to the process by which nitrogen-fixing microorganisms reduce atmospheric N₂ gas into ammonia through nitrogenase (Burris 1991; Rees et al. 2005; Mus et al. 2018). So far, biological nitrogen fixation is found exclusively in diverse prokaryotes including cyanobacteria. Through billions of years of evolution, some autotrophic cyanobacteria have gained the unique capability of using solar energy to aerobically reduce atmospheric N₂ into ammonia through nitrogenase (Fay 1992; Bergman et al. 1997; Berman-Frank et al. 2003; Zehr 2011).

The nitrogenase complex is encoded by a group of genes called *nif* genes, including the most important *nifH*. A minimum of 10 *nif* genes (*nifF*, J, H, D, G, K, U, S, V and B) enable anaerobic nitrogen fixation in an engineered *E. coli* (Yang et al. 2014; Vicente and Dean 2017). The global diversity of nitrogen-fixing microorganisms was assessed through analysis of 16989 *nifH* sequences from available databases (Gaby and Buckley 2011). The soil samples had the greatest diversity of *nifH* sequences among all the environments surveyed. Cyanobacterial *nifH* sequences were also found in topsoil (such as soil crusts) in certain arid ecosystems (Garcia-Pichel et al. 2001; Gaby and Buckley 2011; Hagemann et al. 2015; Zhang et al. 2016). The solar-powered N₂-fixing cyanobacteria in topsoil provides a definite advantage compared to heterotrophic nitrogen-fixing bacteria which fail to use solar energy, relying on other organisms for an energy source; and requiring anaerobic conditions. While this unique phenomenon of N₂-fixing cyanobacteria

has supported the nitrogen needs for native grasslands/forests for eons, mankind has largely ignored its potential application in crop production due to the wide availability of chemically synthesized ammonia driven by fossil fuel energy.

N₂-fixing microbes such as cyanobacteria have played a critical role in healthy soils that harmonize the soil biological, chemical, and physical properties to sustain huge annual biomass production (without applying nitrogen fertilizer) in native grasslands and native forests (Berman-Frank et al. 2003; Adam et al. 2016; Singh et al. 2016). Cyanobacteria can contribute at least 20-30 Kg of fixed N (≈ 55 Kg urea) ha⁻¹ season⁻¹ as well as abundant organic matter to the soil (Pankratova 2006; Issa et al. 2014). The contribution of biomass by cyanobacteria only in arid land soil is estimated to reach 68 x10⁶ tons of carbon, which could greatly improve both soil fertility and soil health (Garcia-Pichel et al. 2003). We hypothesize that the native grassland of South Dakota represents an unexplored territory where highly effective and stress-tolerant nitrogen-fixing cyanobacteria and other N₂-fixing microbes live. The objective of this research was to identify solar-powered, N₂-fixing cyanobacteria and other microbes in topsoil of native grassland in South Dakota. The long-term goal of this project is to engineer these N₂-fixing microbes with the highest capacity for nitrogen fixation and use them as a “solar-powered, living ammonia factory” in crop fields.

METHODS

Isolation and purification of potential N₂-fixing photosynthetic microorganisms. A total of 144 topsoil samples (1-3 cm depth) were collected from native grasslands at the Standing Rock Reservation near McLaughlin, Corson County, SD and native grasslands near Miller, Hand County, SD in May 2016, 2017. Approximately 3-5 grams of homogenized topsoil were added into a 35-ml small mouth bottle filled with 20-ml autoclaved mineralized water (AA/8N medium, a mineralized water without organic carbon sources) with combined nitrogen (Hu et al. 1981). These bottles were incubated under continuous light illumination (ca. 50 $\mu\text{E}/\text{M}^2\cdot\text{S}^{-1}$) at 28-30 °C. After about 10 days, the “green” cultures that appeared were transferred into fresh AA/8N medium to isolate autotrophic microbes from the soil. The green cultures were then streaked onto BG11₀ (free of combined nitrogen) agar plates. After 12 times of re-streaking purification, we isolated six microbial strains that were able to reproducibly grow well on BG110 agar plates, suggesting that these six strains have N₂-fixing ability.

Morphology analysis by microscopy. Microscopy (Olympus, BX53) was used to examine the morphology of isolated cyanobacteria and microalgae.

Nitrogenase activity determined by acetylene reduction assay. A 7-day old culture growing on a BG11₀-agar plate (nitrogen-free medium) was cut and transferred into a 20-ml glass serum bottle (Wheaton). The bottle was sealed with a red rubber stopper (Wheaton) and injected with 1.0 ml of acetylene. The bottle was incubated under continuous light (50 $\mu\text{E}/\text{m}^2/\text{s}$, 120 rpm, 30 °C) for about 3 weeks. Five mL of headspace gas sample from the 20 mL culture bottle was

administered via a 1 mL GSV Loop to the GC-MS (Agilent 890A/5975C). To alleviate the vacuum in the serum bottle, we injected 4 mL of normal air and 1 mL CO₂ back into the 20 mL bottle. The volatile compounds were separated by CP7348 column (Agilent PoraBOND Q 25 m × 250 μm × 3 μm) with Pulsed Split mode at 20:1 ratio at a flow rate 0.8 mL/min using hydrogen as a carrier gas. The GC program was initiated at 32 °C held for 4 min, and ramped at 110 °C to reach 232 °C. The scanning mass range of MSD was between 10 to 50 m/z.

RESULTS AND DISCUSSION

Isolation of four microalgae strains. Four photosynthetic micro-organisms were isolated from topsoil of native grassland in South Dakota. These four microbial strains reproducibly grew well autotrophically in the nitrogen-free medium plate (Figure 1, left panels) after more than 10 times re-streaking onto the nitrogen-free medium-BG110-agar plate (Figure 1). Xu15, Xu81 and Xu111 are ellipsoidal in shape (Figure 1, right panels), while Xu141 is spherical in shape (Figure 1). Based on their cell sizes (5.0-10 μm) and other visual microscopic morphology such as compartmentalized organelles, Xu15, Xu81, Xu111 and Xu141 are likely unicellular microalgae (Figure 1, right panels). Microalgae are unicellular microorganisms, having many compartmentalized organelles such as a nucleus, mitochondria and chloroplast within each cell; while cyanobacteria are usually multi-cellular microorganisms, although some cyanobacteria are unicellular organisms. Unlike microalgae, cyanobacteria lack a nucleus and mitochondria. Both microalgae and cyanobacteria can perform plant-type photosynthesis by using water as a source of electrons and releasing oxygen.

Isolation of two filamentous cyanobacterial strains. Two filamentous cyanobacterial strains were isolated from the topsoil of native grasslands in South Dakota. These two isolates reproducibly grew well autotrophically in the nitrogen-free medium-BG11₀-agar plate (data not shown), which suggests they are capable of nitrogen fixation. The cell size of strain Xu86 was in the range of 1.0-2.0 μm (Figure 2, Xu86 panel), while the cell size of strain WW3 was in the range of 2.0-3.0 μm (Figure 2, WW3 panel). Both Xu86 and WW3 strains had substantial motility on nitrogen-free medium-BG11₀-agar plates (data not shown).

Nitrogenase activity of culture Xu15 and culture Xu81. Since the culture of Xu15 and Xu81 appeared to be homogeneous at least microscopically, and given that they can grow well on the nitrogen-free medium, this strongly suggests that these two microalgae may have nitrogenase activity. The acetylene reduction assay was used to determine the nitrogenase activity for cultures Xu15 and Xu81. Both cultures showed a substantial ethylene production (Figure 3, C & D) after about 3 weeks of incubation with acetylene; while the controls with non-N₂-fixing cyanobacterium *Synechocystis* sp. PCC 6803 showed no ethylene production in conditions identical to that of Xu15 and Xu81 (data not shown). The substantial ethylene production from the cultures of Xu15 and Xu81 suggests the presence of

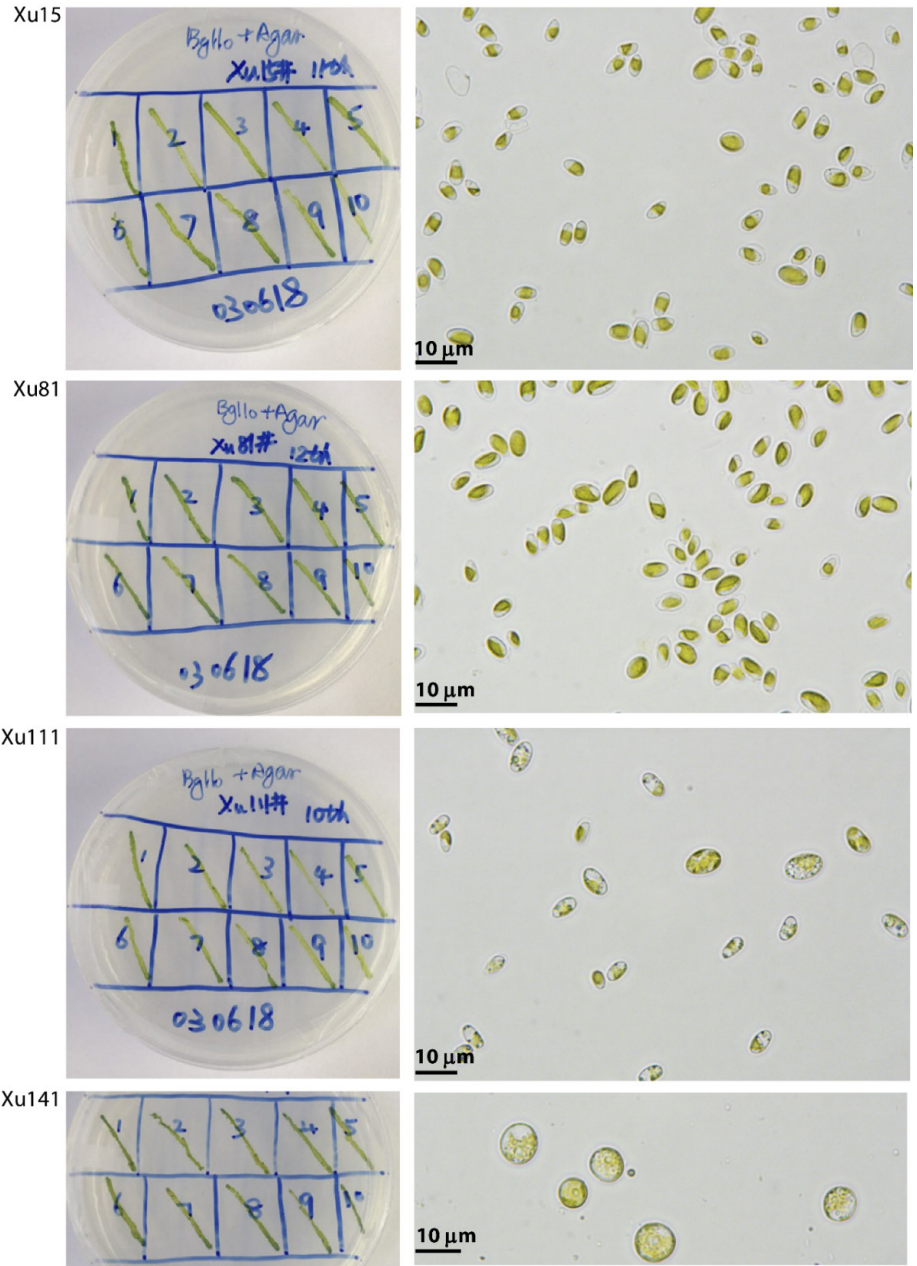
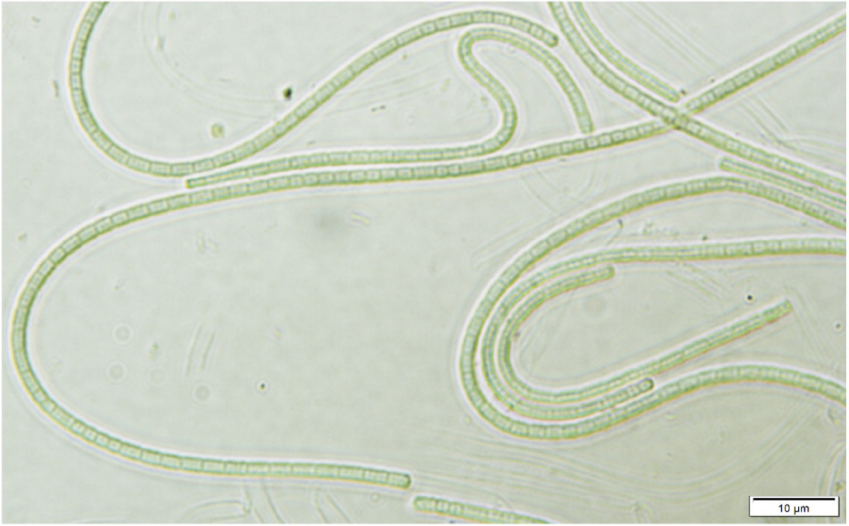


Figure 1. Four microalgae isolated from the topsoil of native grasslands in South Dakota. Left panels: four microalgae grown on BG11_σ-agar plate (nitrogen-free medium). Right panels: bright-field images captured by an Olympus BX53 upright microscopy equipped with a digital camera.

Xu86



WW3

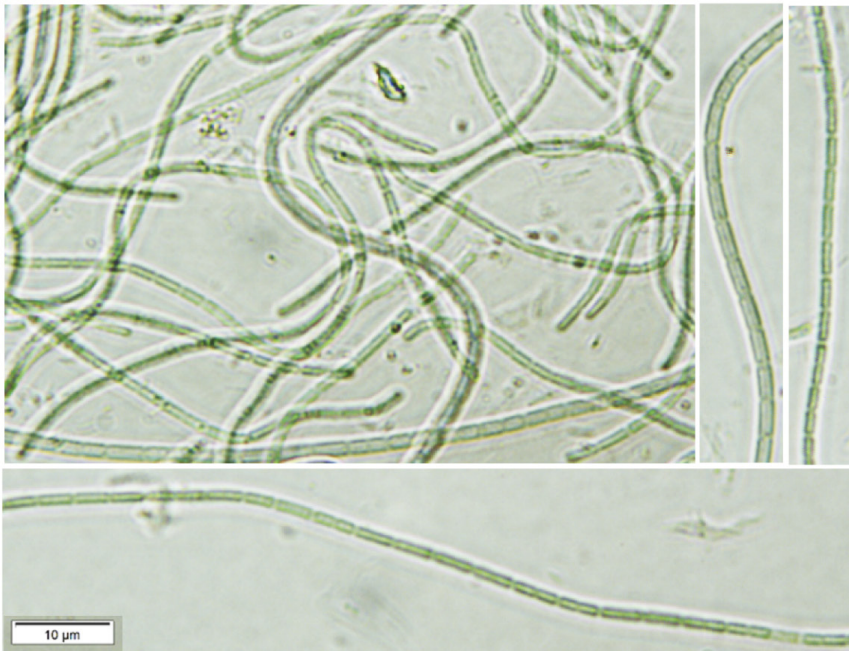


Figure 2. Two filamentous cyanobacterial strains isolated from the topsoil of native grasslands in South Dakota. These strains were able to grow well on free of combined nitrogen medium. Bright-field images for both Xu86 and WW3 grown in AA/8N (Hu et al. 1981) were captured by an Olympus BX53 upright microscopy equipped with a digital camera. The scale bars in all panels including 4 panels for WW3 were 10 μm.

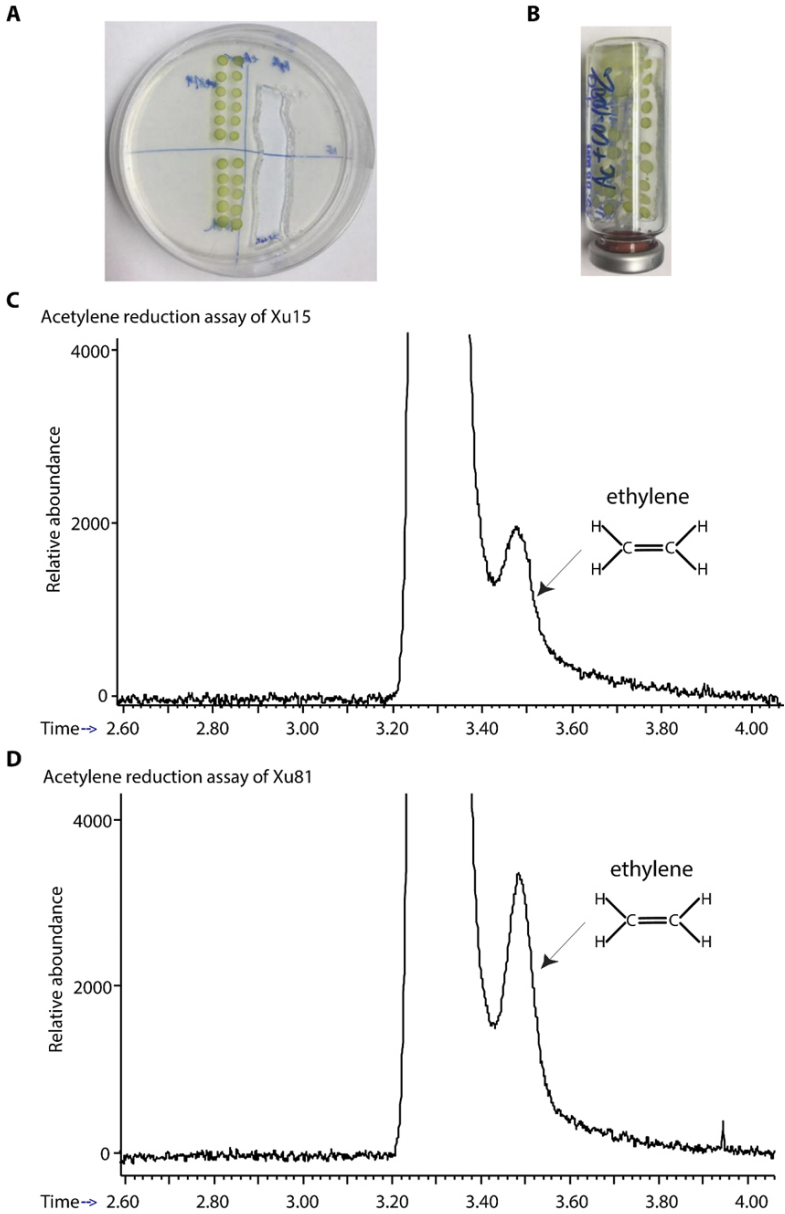


Figure 3. Acetylene reduction assay for cultures of Xu15 and Xu81 grown on BG11₀-agar plate (nitrogen-free medium). A) Xu15 or Xu81 culture incubated for about 7 days on BG11₀-agar plate under continuous light illumination (ca. 50 μEIM⁻²S⁻¹) at 28-30 °C. B) The culture along with the agar transferred into a 20 mL clear serum bottle and sealed with rubber stopper. C) Ethylene peak detected in the headspace gas sample of culture Xu15. D) Ethylene peak detected in the headspace gas sample of culture Xu81.

nitrogenase activity in these two cultures. However, the 18S rRNA sequences that we obtained identified Xu15 and Xu81 to be *Chloroidium saccharophilum*, a common terrestrial coccoid green alga (Darienko et al. 2010). Since there has been no report so far that *Chloroidium saccharophilum*, a eukaryotic microorganism, has nitrogenase activity, additional investigation and more evidences are required to further validate the nitrogen fixation ability of *Chloroidium saccharophilum* strains Xu15 and Xu81 that were isolated from South Dakota native grasslands.

Partial genome sequencing of Xu15 strain to obtain its 18S rRNA gene sequence. Very recently we started to sequence the genome for strain Xu15. The 18S rRNA gene containing contig_85 (bp 6718 to bp 10208) from Xu15 genomic DNA showed the highest identity (99.50% identical) to the partial 18S rRNA gene-containing sequences (GenBank access #: KC517116.1, KX024689.1) from both the *Chloroidium saccharophilum* strain CCM-UDEC-143 and the *Chloroidium saccharophilum* strain SHY184 (Song et al. 2016), a common terrestrial coccoid green algae (Darienko et al. 2010). The 18S rRNA gene sequence alignment among Xu15 and the two *Chloroidium saccharophilum* strains is shown in Figure 4. Based on the cell morphology and its 18S rRNA sequence data, we conclude that Xu15 belongs to *Chloroidium*. Therefore, strain Xu15 was reassigned *Chloroidium saccharophilum* Xu15. This newly isolated *Chloroidium saccharophilum* Xu15 strain will be stored at ATCC (the American Type Culture Collection). The 18S rRNA gene sequence will be deposited at GenBank.

Conclusion. Six photosynthetic microorganisms were isolated from native grasslands in South Dakota. These six isolates were all capable of growing autotrophically in nitrogen-free medium, which strongly suggests that these six isolates may have the ability to fix N₂ aerobically. Acetylene reduction assay detected a substantial production of ethylene, suggesting a nitrogenase activity for cultures Xu15 and Xu81. Four of the isolated strains (Xu15, Xu81, Xu111, Xu141) appeared to be unicellular microalgae. Based on its 18S rRNA gene sequence that we obtained, strain Xu15 was reassigned as *Chloroidium saccharophilum* Xu15 strain. Xu81 looks identical to Xu15. Both Xu86 and WW3 are likely filamentous cyanobacteria. To our best knowledge, this is the first successful isolation of photosynthetic micro-organisms from topsoil of South Dakota native grasslands. Future research will focus on validation and developing these validated N₂-fixing microbes as *in situ*, solar-powered living ammonia factories in crop fields.

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KX024689.1	TGTATAGTATTTGATGGTACCTACTACCGGTAACCGTAAACGAGAGTATTGGCGTAGGGAGACAGAGAGTGCCTGATGGCT								
consensus>50	tagtcatatgcttgtctcaaaagattagccatgcgatgctaaagataaactgcttactcggaactgccaatggctcattaataca								
	100	110	120	130	140	150	160	170	
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KX024689.1	GTATAGTATTTGATGGTACCTACTACCGGTAACCGTAAACGAGAGTATTGGCGTAGGGAGACAGAGAGTGCCTGATGGCT								
consensus>50	gtatagtttatttgatggtaacctactaccggtaaccgtaaacgagagtattggcgtagggagacagagagtgccttagtgct								
	180	190	200	210	220	230	240	250	260
Kx15_6718-1208	GGCAGAGCTGGCAGACTGTCAAATGCGTGCATCCCGCTACGCTGAGGAGACCGCTGATGGGAAAACCTGATCGGCACCGCAG								
KC517116.1	GGCAGAGCTGGCAGACTGTCAAATGCGTGCATCCCGCTACGCTGAGGAGACCGCTGATGGGAAAACCTGATCGGCACCGCAG								
KX024689.1	GGCAGAGCTGGCAGACTGTCAAATGCGTGCATCCCGCTACGCTGAGGAGACCGCTGATGGGAAAACCTGATCGGCACCGCAG								
consensus>50	gctcagagctggcagactgtcnaatgctgctgacatcccgctacgctgaggagaccgctgattgggaaaacctgatcggcacccgag								
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KX024689.1	GGGAAAGCGCGGGTATGTGTAACACTCTCTCGGCTAGGGACTATGGGAGCAGAGCTCAAAGGCGCTTGGCTCAAGAGTGCAG								
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consensus>50	caaattaccaatcccgaccgggaggtagtgacaataaacaataccgggcttttcaagctcgttaattggaatgagtacaat								
	890	900	910	920	930	940	950	960	
Kx15_6718-1208	CTAAATCCCTTAACGAGGATCAATGGAGGCAAGTCTGGTAACTGTTTCAGCAAGCTTCCAGCTGTGACGCCAGAGATAGCGGG								
KC517116.1	CTAAATCCCTTAACGAGGATCAATGGAGGCAAGTCTGGTAACTGTTTCAGCAAGCTTCCAGCTGTGACGCCAGAGATAGCGGG								
KX024689.1	CTAAATCCCTTAACGAGGATCAATGGAGGCAAGTCTGGTAACTGTTTCAGCAAGCTTCCAGCTGTGACGCCAGAGATAGCGGG								
consensus>50	ctaaatcccttaacgagatcaatggaggcaagtctggttaactgtttcagcaagcttccagctgtgacgccagagatagcgggg								
	970	980	990	1000	1010	1020	1030	1040	1050
Kx15_6718-1208	CGGACCCGAGGAGCTCGGGCGCTAAGCGTGTAGCTGTGACGACCAACACTGGATCGGGTGGTCCGGCAGAGCAGCTTGGAA								
KC517116.1	CGGACCCGAGGAGCTCGGGCGCTAAGCGTGTAGCTGTGACGACCAACACTGGATCGGGTGGTCCGGCAGAGCAGCTTGGAA								
KX024689.1	CGGACCCGAGGAGCTCGGGCGCTAAGCGTGTAGCTGTGACGACCAACACTGGATCGGGTGGTCCGGCAGAGCAGCTTGGAA								
consensus>50	cggaccgagagactcggcgctaaagcgtgtagctgtgacgaccaaacactggatcgggtggtcggcagagcagcttggaa								
	1060	1070	1080	1090	1100	1110	1120	1130	1140
Kx15_6718-1208	GGGCAAGCGCTCACTAGAGGCACTCTCAGCGGCTGATCCCGTGGGAGCTCCAGGGTGACCCTCTCAGGAGCGGCTGTAAC								
KC517116.1	GGGCAAGCGCTCACTAGAGGCACTCTCAGCGGCTGATCCCGTGGGAGCTCCAGGGTGACCCTCTCAGGAGCGGCTGTAAC								
KX024689.1	GGGCAAGCGCTCACTAGAGGCACTCTCAGCGGCTGATCCCGTGGGAGCTCCAGGGTGACCCTCTCAGGAGCGGCTGTAAC								
consensus>50	gggcaagcgcctcactagagcactctcagcggctgatcccgtaggagctccagggtgaccctctcaggagcggctgtaac								
	1150	1160	1170	1180	1190	1200	1210	1220	1230
Kx15_6718-1208	GCACCGTAAGCGCTCGGCTCGGCTCAGGCACTCCGCTAAGGAGCACTCTAATCCAGCGGATGATAAAGCGTCTTGGGCTG								
KC517116.1	GCACCGTAAGCGCTCGGCTCGGCTCAGGCACTCCGCTAAGGAGCACTCTAATCCAGCGGATGATAAAGCGTCTTGGGCTG								
KX024689.1	GCACCGTAAGCGCTCGGCTCGGCTCAGGCACTCCGCTAAGGAGCACTCTAATCCAGCGGATGATAAAGCGTCTTGGGCTG								
consensus>50	gcaccgtaagcgcctcggctcggctcaggcactccgctaaaggagcactctaataccagcggatgataaagcgtcttgggctg								
	1240	1250	1260	1270	1280	1290	1300	1310	1320
Kx15_6718-1208	GAGACCCGAGTCTCGAATCGTCAAGGAGCGGCGCTGGCAAGGCGCAGCGGCTCGGAGTAAATGCCAGAGCGGGGTAATPCCA								
KC517116.1	GAGACCCGAGTCTCGAATCGTCAAGGAGCGGCGCTGGCAAGGCGCAGCGGCTCGGAGTAAATGCCAGAGCGGGGTAATPCCA								
KX024689.1	GAGACCCGAGTCTCGAATCGTCAAGGAGCGGCGCTGGCAAGGCGCAGCGGCTCGGAGTAAATGCCAGAGCGGGGTAATPCCA								
consensus>50	gagaccgagtcgtaactcgaatcgtcaaggagcggcgctggcaaggcgcagcggctcggagtaaatgccagagcgggtaatpcca								
	1330	1340	1350	1360	1370	1380	1390	1400	
Kx15_6718-1208	GCTCCATAGCGTATTTAAGTGTGTGAGTAAAAAAGCTCGACTTGGATTTGGGCGGGGCGCTCGGCTCCGCTGCTGTGGGCA								
KC517116.1	GCTCCATAGCGTATTTAAGTGTGTGAGTAAAAAAGCTCGACTTGGATTTGGGCGGGGCGCTCGGCTCCGCTGCTGTGGGCA								
KX024689.1	GCTCCATAGCGTATTTAAGTGTGTGAGTAAAAAAGCTCGACTTGGATTTGGGCGGGGCGCTCGGCTCCGCTGCTGTGGGCA								
consensus>50	gctccatagcgtatattagtggtgtgagtaaaaaagctcgcacttggatttggcgggcgctcggctccgctcctgtgggca								
	1410	1420	1430	1440	1450	1460	1470	1480	1490
Kx15_6718-1208	CTGGCATGGGCTCGGCTCGGCTCGGCGGAGCGGCTCGGCTTAACTGTCCGGGACTCGGACTCGACAGGTTTACTTTCGATTAAT								
KC517116.1	CTGGCATGGGCTCGGCTCGGCTCGGCGGAGCGGCTCGGCTTAACTGTCCGGGACTCGGACTCGACAGGTTTACTTTCGATTAAT								
KX024689.1	CTGGCATGGGCTCGGCTCGGCTCGGCGGAGCGGCTCGGCTTAACTGTCCGGGACTCGGACTCGACAGGTTTACTTTCGATTAAT								
consensus>50	ctggcatgggctcggctcggctcggcgagcggctcggcttaaactgtccgggactcggactcgacaggtttactttcgattaat								
	1500	1510	1520	1530	1540	1550	1560	1570	1580
Kx15_6718-1208	AGAGTGTCAAAGCAGGCTACGCTCGAATAGTATAGCATGGAAATACAGATAGGACCTCGGCTATCTTGTGGTGTGGGAC								
KC517116.1	AGAGTGTCAAAGCAGGCTACGCTCGAATAGTATAGCATGGAAATACAGATAGGACCTCGGCTATCTTGTGGTGTGGGAC								
KX024689.1	AGAGTGTCAAAGCAGGCTACGCTCGAATAGTATAGCATGGAAATACAGATAGGACCTCGGCTATCTTGTGGTGTGGGAC								
consensus>50	agagtgtcaaacagcgcctacgctcgaatagtatagcatggaaatacagataggacctcggctatcttgtgggtgggacc								
	1590	1600	1610	1620	1630	1640	1650	1660	1670
Kx15_6718-1208	GGAGTAATGATTAAGAGGAGCGGCTCGGGGCACTCGTATTTCAATGTGAGAGTAAATCTTGGATTTAAGAAAGCAAGCTCTCG								
KC517116.1	GGAGTAATGATTAAGAGGAGCGGCTCGGGGCACTCGTATTTCAATGTGAGAGTAAATCTTGGATTTAAGAAAGCAAGCTCTCG								
KX024689.1	GGAGTAATGATTAAGAGGAGCGGCTCGGGGCACTCGTATTTCAATGTGAGAGTAAATCTTGGATTTAAGAAAGCAAGCTCTCG								
consensus>50	ggagtaatgattaaaggagcggctcggggcactcgtatttcaatgtgagagtaaatcttggatttaagaaagcaagctctcg								
	1680	1690	1700	1710	1720	1730	1740	1750	1760
Kx15_6718-1208	GAAAGCATTTGCCAAGGATGTTTCAATTAACAGAACGAAAGTGGGGGCTCGAAGCAGATATAGTACCGCTCTAGCTCCAACCAATA								
KC517116.1	GAAAGCATTTGCCAAGGATGTTTCAATTAACAGAACGAAAGTGGGGGCTCGAAGCAGATATAGTACCGCTCTAGCTCCAACCAATA								
KX024689.1	GAAAGCATTTGCCAAGGATGTTTCAATTAACAGAACGAAAGTGGGGGCTCGAAGCAGATATAGTACCGCTCTAGCTCCAACCAATA								
consensus>50	gaaagcatttgccaaggattgtttcaatatacagaacgaaagtgggggctcgaagcagattagtagccgctctagctccaaccaata								

