

1 Original Research Article
2 **Evaluation of Antibiotic Biosynthetic Potential**
3 **of Actinomycete Isolates to Produce**
4 **Antimicrobial Agents**

5
6
7
8
9
10 **ABSTRACT**
11

Aim: To assess the antibiotic biosynthetic potential of actinomycete isolates from rhizospheric soil samples collected from Ngaka Modiri Molema district in North West Province of South Africa.

Study design: The analysis of biosynthetic gene clusters through PCR-based approach presents a useful foundation for the discovery of bioactive compounds.

Place and Duration of Study: Microbial Biotechnology Laboratory, Department of Biological Sciences, North-West University, Mafikeng Campus, South Africa, between June 2011 and November, 2013.

Methodology: Through PCR-based approach 341 actinomycete isolates were screened for the polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) biosynthetic gene clusters. The amplification of the genes from some of the actinomycete isolates is an indication of their potential as antibiotic producers. Phylogenetic analysis using PKS-I, PKS-II and NRPS gene sequences were conducted.

Results: Sixteen isolates (4.69%) were identified as PKS-I gene positive strains, 15.25% for PKS-II and 13.48% for NRPS gene. Through the screening, it was found that *Streptomyces* have higher prevalence of PKS-I, PKS-II and NRPS genes compared to others genera. Phylogenetic analysis of the nucleotide sequences from the amplified biosynthetic genes confirmed that the isolates formed a close phylogenetic relationship with known antibiotic producers.

Conclusion: PCR-based approach using degenerative primers to screen for the presence of biosynthetic gene clusters responsible for the biosynthesis of bioactive secondary metabolites, is an effective approach for discovering diverse antibiotics from actinomycetes.

12
13 *Keywords: Actinomycetes, secondary metabolites, biosynthetic gene cluster, PCR screening*
14 *PKS, NRPS*

15 **1. INTRODUCTION**
16

17 Terrestrial actinomycetes are well known as prolific producers of secondary metabolites.
18 These secondary metabolites exhibit pharmaceutical properties, such as antibacterial,
19 antifungal, antiprotozoal, anticancer, anticholesterol and immunosuppressant. These
20 bioactive compounds were synthesized through biosynthetic metabolic pathways. Polyketide
21 synthases (PKS) and non-ribosomal peptide synthetases (NRPS) pathways are synthesized
22 by various specific enzymatic steps [1]. These enzymes perform the repetitive chemical
23 condensation of monomeric units, such as carboxylic acid and/or amino acid monomers. The
24 vast structural complexity and functional diversity of PKS, NRPS or hybrid compounds are
25 as a result of complex enzyme organization of the assembly lines and specific tailoring

enzymes responsible for post-assembly modifications [2]. Recent advances in the areas of small molecule detection, isolation and structure elucidation, genome sequencing and molecular techniques have helped improve the understanding of the molecular genetics of secondary metabolite biosynthesis, rekindling interest in discovery platforms aimed at realizing the biosynthetic capacity of antibiotic producing microorganisms [2].

The PCR based approaches have been used successfully to amplify genes associated with secondary metabolite biosynthesis and therefore it is possible to predict whether secondary metabolite pathways are present within an organism [3]. It becomes feasible to envisage or estimate the number and novelty of bioactive compounds following phylogenetic analysis of the amplified and sequenced secondary metabolite genes. Secondary metabolites through PKS and NRPS pathways have proven to be one of the primary sources of new chemical scaffolds from which novel pharmaceutical agents have been developed [4].

The order *Actinomycetales* are renowned producers of bioactive metabolites with a track record of over 10,000 antimicrobial agents in clinical use [5]. Biosynthesis of these secondary metabolites is catalyzed by specific enzymes usually encoded by gene clusters. Polyketide synthases and non-ribosomal peptide synthetases are the major enzymes of secondary metabolites synthesis [6]. Examples of classes of antibiotics produced through this biosynthesis include ansamycins, tetracyclines, polyenes and glycopeptides [7].

In an effort to discover potentially novel bioactive compounds, degenerate primer sets were used to screen for the presence of biosynthetic gene clusters associated with PKS-I, PKS-II and NRPS pathways in genomic DNA of 341 actinomycete strains. The antibacterial potential of the actinomycete strains was based on the amplification of the expected size of the gene of interest. The PCR screening of the genomic DNA for specific antibiotic genes of interest allows for the rapid determination of the antibiotic biosynthetic potential of the isolates.

2. METHODOLOGY

2.1 Bacterial strains and cultivation

All actinomycete strains isolated and identified from rhizospheric soil samples were grown in Luria Bertani broth (Merck) at 30°C with agitation in shaker incubator for 7 days [8].

2.2 Antibacterial assays

Antagonistic activity of actinomycete isolates against Gram-negative and Gram-positive bacteria was screened by using perpendicular streak method [8, 9]. In perpendicular streak method, Mueller Hinton agar (Merck) was used and each plate was streaked with individual actinomycete isolates at the center/diameter of the plate and incubated at 30°C for 7 days. Later, 24 h fresh sub-cultured test bacteria were prepared and streaked perpendicular to the isolates and incubated at 37°C for 24 h. The experiment was carried out in triplicate.

2.3 DNA extraction

The genomic DNA was extracted from all the actinomycete isolates using the cetyltrimethyl ammonium bromide (CTAB) method as previously described [10].

2.4 PCR primers

68 Four sets of primers were used: F1: 5'-AGAGTTTGATCITGGCTCAG-3' and R5: 5'-
69 ACGGITACCTTGTTACGACTT-3' targeting 16S rDNA gene [11]. AHBA-F: 5'-
70 CCSGCSTTCACSTTCATCTC-3' and AHBA-R: 5'-AISYGGAICATIGCCATGTAG-3' targeting
71 3-amino-5-hydroxyl-benzoic acid (AHBA) synthase gene [3]. ARO-PKS-F: 5'-
72 GGCAGCGGITTCCGGCGITTCCAG-3' and ARO-PKS-R: 5'-
73 CGITGTTIACIGCGTAGAACCAGGCG-3' targeting the ketosynthase alpha (KS_{α}) and
74 ketosynthase beta (KS_{β}) gene pair [3]. NRPS-A3F: 5'-GCSTACSYSATSTACACSTCSGG-3'
75 and NRPS-A7R: 5'-SASGTCVCCSGTSCGGTAS-3' targeting the targeting NRPS
76 adenylation (A) domains [12]. All the oligonucleotide primers were synthesized by Integrated
77 DNA Technologies (South Africa).

78 2.5 PCR amplification conditions

79 PCR was performed in a total volume of 50 μ l containing 30-50 ng DNA, 100 mM of each
80 primer, 0.05 U/ μ l *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP
81 (Fermentas, USA). The amplification reaction was performed with a DNA Engine DYAD
82 Peltier thermal cycler (BioRad, USA). The PCR cycling programme was as follows: initial
83 denaturation (96°C for 2 min); 30 cycles of denaturation (96°C for 45 s), annealing (56°C for
84 16S rRNA and AHBA synthase genes/64°C for the KS_{α} - KS_{β} gene pair/57°C for NRPS gene,
85 for 30 s), and extension (72°C for 2 min); and a final extension (72°C for 5 min). The PCR
86 amplicons were analyzed by electrophoresis on 1% (w/v) agarose gel. The gel containing
87 ethidium bromide (10 μ g/ml) was viewed under Syngene Ingenius Bioimager (UK) to confirm
88 the expected size of the PCR products. The remaining mixture was purified using
89 NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Germany).

90 2.6 DNA sequencing

91 Sequencing of the purified PCR products was conducted at the facilities of Inqaba
92 Biotechnical Industries (Pty) Ltd, Pretoria, South Africa using ABI PRISM® 3500XL DNA
93 Sequencer (Applied Biosystems, USA).

94 95 2.7 Phylogenetic analysis

96 All nucleotide sequences for the 16S-rDNA, PKS and NRPS phylogenetic trees were
97 obtained from GenBank, except for the sequences that were determined in this study.
98 Nucleotide sequences were analyzed and edited using BioEdit software package version
99 7.1. The obtained sequences were compared to sequences in the NCBI GenBank database
100 with the Basic Local Alignment Search Tool (BLAST) [13]. Multiple alignments of the
101 sequences were carried out by Mafft program 7.050 [14] against corresponding nucleotide
102 sequences retrieved from GenBank. Phylogenetic and molecular evolutionary analyses were
103 conducted using software in MEGA version 5.2.2 [15]. Evolutionary distance matrices were
104 generated and a phylogenetic tree was inferred by neighbor-joining method [16]. Tree
105 topologies were evaluated by bootstrap analysis [17] based on 1000 resamplings of the
106 neighbor-joining data set. Manipulation and tree editing were carried out using TreeView
107 [18].
108

109 2.8 Nucleotide sequence accession numbers

110 The GenBank accession numbers of the nucleotide sequences generated in this study are
111 shown in parentheses beside the isolate codes on the phylogenetic trees.
112

113 3. RESULTS

114

115 3.1 Bioactivity of actinomycete isolates

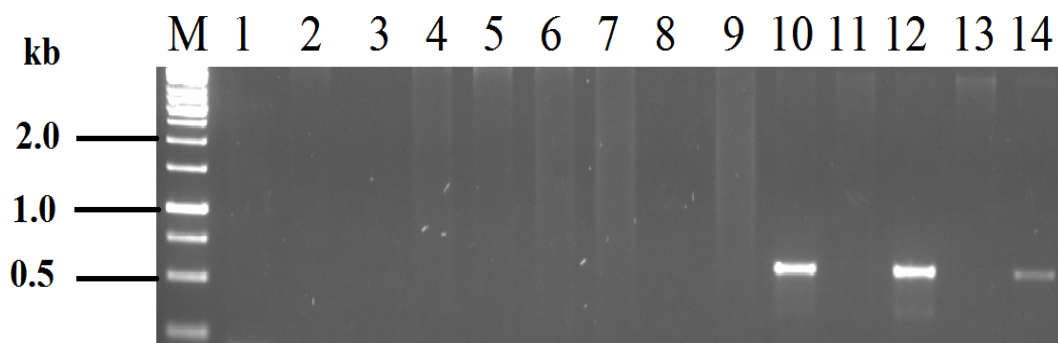
116 All the actinomycete isolates were tested for antibacterial activity using cross streak method.
117 The result shows that 54% of the isolates exhibited antibacterial activity against one or more
118 of the test organisms [8]. Analysis of the bacterial isolates showed that the genus
119 *Streptomyces* is the most active in exhibiting broad spectrum antimicrobial activities [19].

120 3.2 Detection of biosynthetic gene in actinomycete isolates

121 Degenerate primers targeting genes encoding for polyketide synthases (PKS-I and PKS-II)
122 and non-ribosomal peptide synthetases (NRPS) were used to screen the biosynthetic
123 potential of 341 actinomycete isolates, as identification of these genes provides indirect
124 evidence of potential chemical diversity among these actinobacteria in terms of natural
125 product drug discovery. The antibiotic producing actinomycete isolates were the subject of
126 16S rDNA gene sequencing which is used for the identification of the strains by BLAST
127 search program. It is apparent that the actinomycete isolates can be assigned to the genera
128 *Streptomyces* spp, *Nocardia* spp, *Rhodococcus* spp, *Pseudonocardia* spp, *Saccharothrix*
129 spp, *Promicromonospora* spp, *Arthrobacter* spp, *Micrococcus* spp, *Nocardiopsis* spp,
130 *Streptosporangium* spp, *Nonomuraea* spp and *Actinomadura* spp.

131 PCR screening for the biosynthetic gene involved in the production of antimicrobial agents
132 depends on the amplification of the predicted size of the DNA fragments. As expected, PCR
133 screening of the genome of the actinomycete isolates for the biosynthetic gene yielded DNA
134 fragments of the predicted size from the potential producers. PCR screening for the PKS-I,
135 yielded DNA fragments of approximately 500 bp (Fig. 1). The results indicate 4.69% (16)
136 positive strains for the presence of PKS-I biosynthetic gene cluster from the 341
137 actinomycete isolates screened. Molecular identification of the actinomycete isolates using
138 16S rDNA shows that *Streptomyces* are the most prominent genus containing the AHSA
139 biosynthetic gene. A standard nucleotide BLAST search was carried out using the partial
140 nucleotide sequences of the positive strains against reference sequences in the GenBank
141 database. This showed that the actinomycete isolates were most similar to other PKS-I
142 producers such as *Amycolatopsis mediterranei*, *Micromonospora* spp, and *Streptomyces*
143 spp. Fig. 2 shows the results of the PCR screening for the PKS-II gene pair, KS_α and KS_β
144 with an expected amplified DNA fragment of approximately 500 bp. Out of 341 actinomycete
145 isolates screened 15.25% were found to contain the biosynthetic gene for PKS-II. A BLAST
146 search against the GenBank database using the nucleotide sequences generated from this
147 study showed that the positive strains were most similar to the corresponding sequences
148 from aromatic polyketide producers. An approximately 750 bp DNA fragment was amplified
149 from the positive strains for NRPS gene (Fig. 3). Of all the 341 actinomycete isolates
150 screened, 13.48% (46) were positive for the presence of the NRPS pathway. The NRPS
151 gene sequences were generated and compared to sequences in the GenBank by BLAST
152 analysis. The result showed that majority of the strains belong to the genus *Streptomyces*.

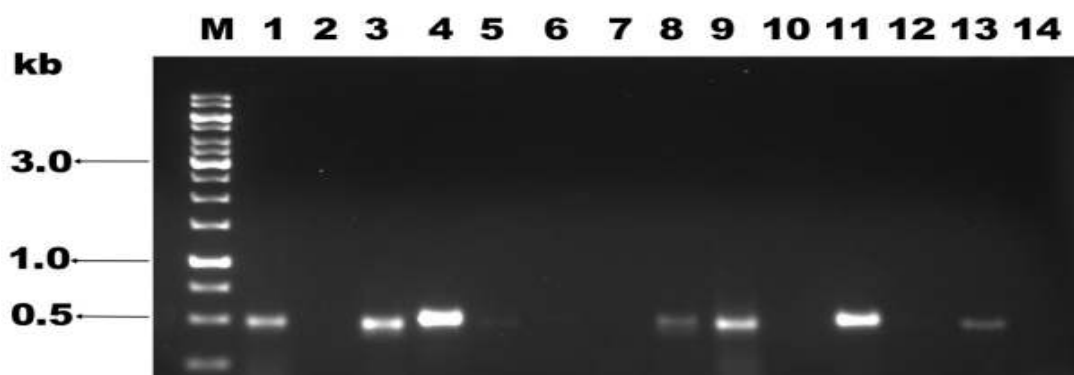
153



154

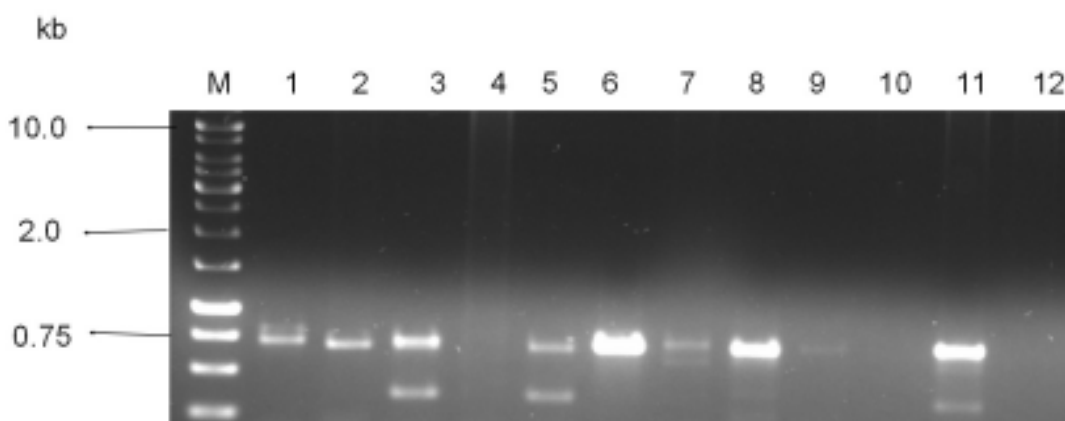
155 **Figure 1: Agarose gel electrophoresis of PCR products from selective amplification of**
 156 **500 bp fragment using AHBA-F/AHBA-R specific for PKS-I sequences**

157



158

159 **Figure 2: Agarose gel electrophoresis of PCR products from selective amplification of**
 160 **500 bp fragment using ARO-PKS-F/ARO-PKS-R specific for PKS-II sequences**



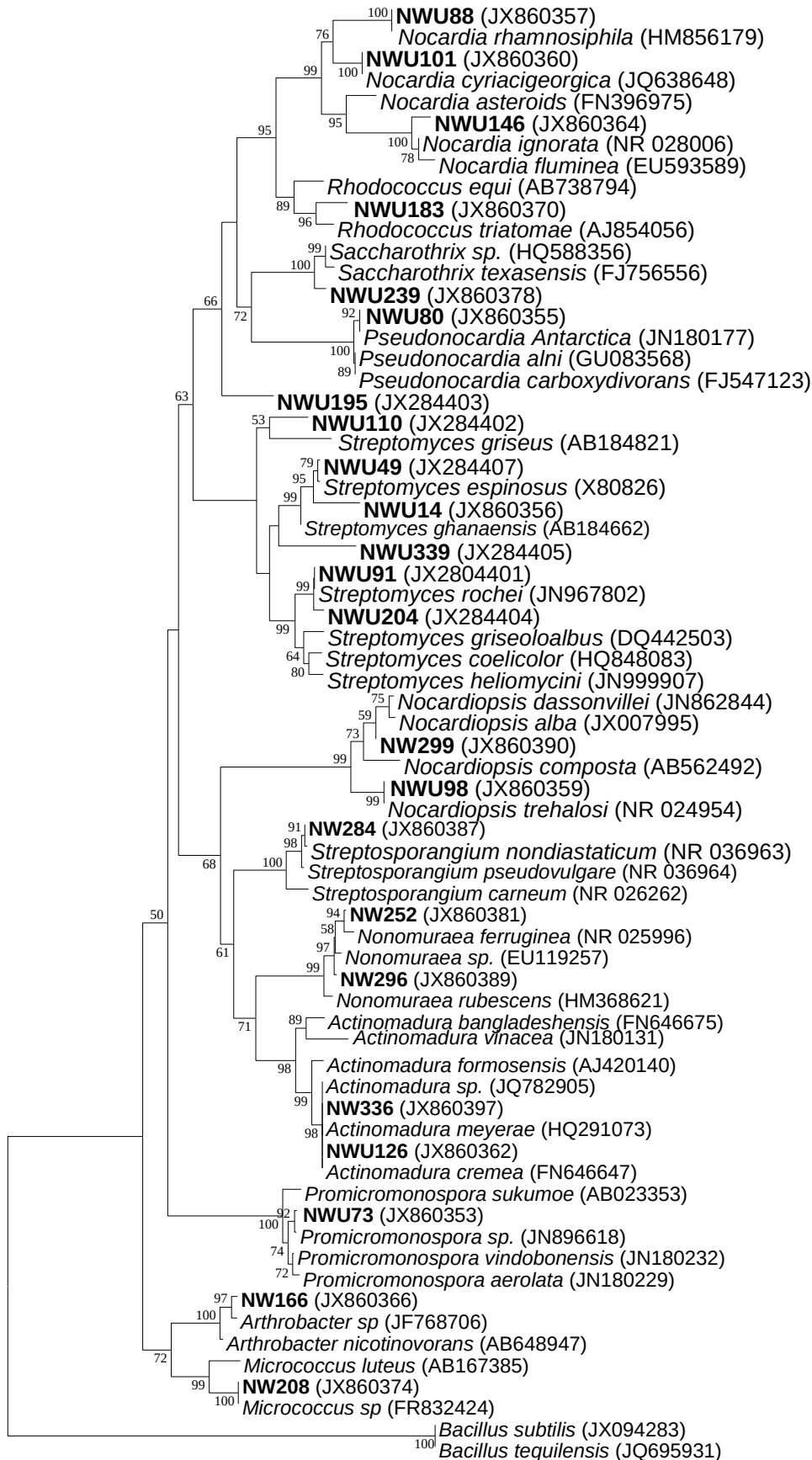
161

162 **Figure 3: Agarose gel electrophoresis of PCR products from selective amplification of**
 163 **750 bp fragment using NRPS-A3F/NRPS-A7R specific for NRPS adenylation**
 164 **sequences**

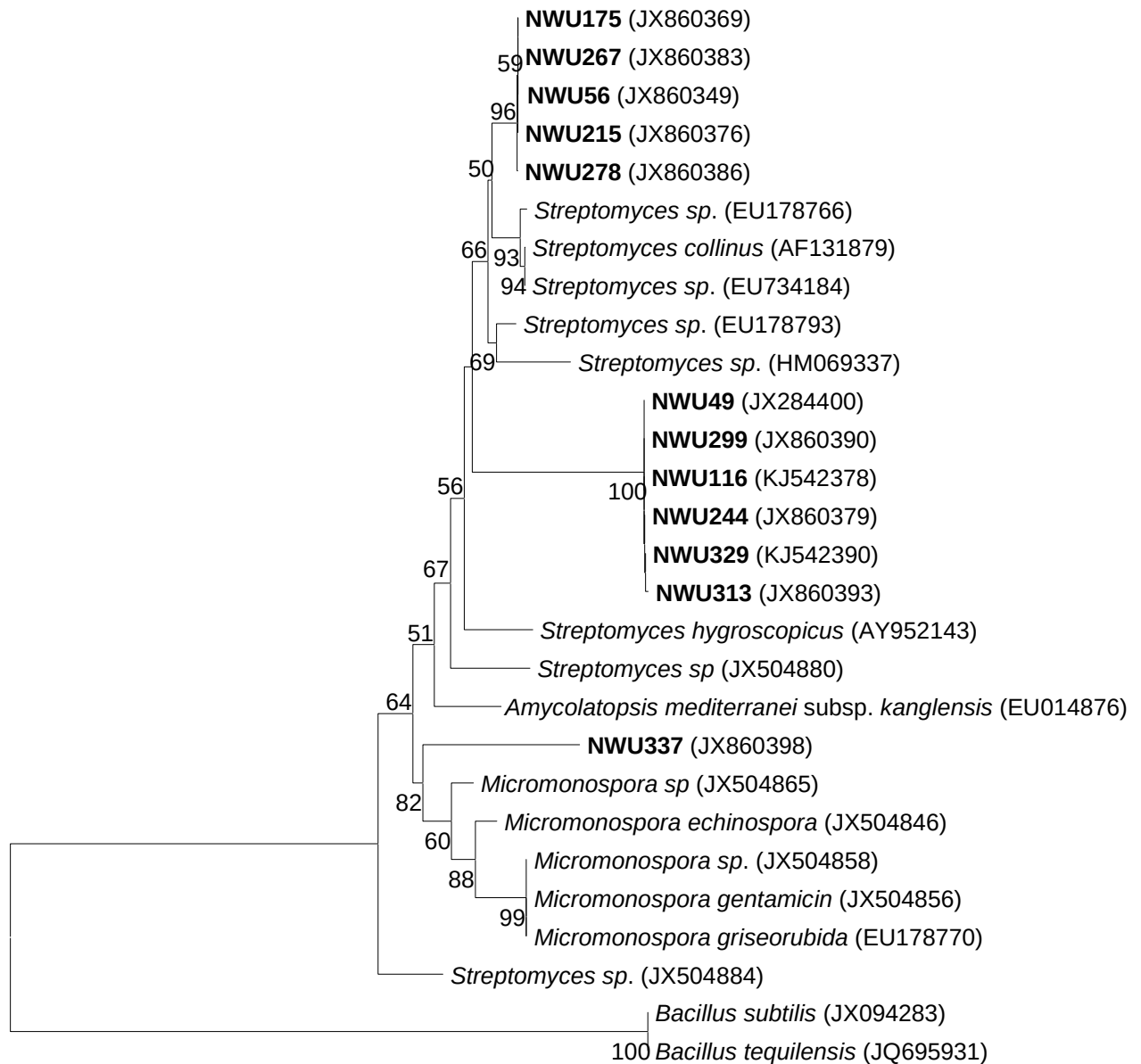
165 3.3 Phylogenetic analysis of antibiotic producing actinomycete isolates

166 The phylogenies based on the 16S rDNA and biosynthetic gene nucleotide sequences from
167 the actinomycete isolates constructed by the neighbor-joining method are shown in Fig. 4-7.
168 Trees constructed by the UPGMA and maximum parsimony method gave similar topology
169 (data not shown). Actinomycete isolates for the 16S rDNA were picked for the phylogenetic
170 tree based on the exhibition of inhibitory activities against at least one test organism.
171 Analysis based on the 16S rDNA gene alignments showed that the actinomycete isolates
172 shared between 89-100% similarities with their closest relatives in the GenBank database
173 that are known antimicrobial agent producers. It was also found that the actinomycete
174 isolates formed a clade or cluster with already known antibiotic producing actinomycete
175 reference sequences from the GenBank. As seen from the phylogenetic tree (Fig. 4),
176 depicting also bootstrap values, the 23 antibiotic producing actinomycete isolates were
177 sorted into 14 main clusters with highest similarity to already known antibiotic producing
178 actinomycete reference sequences from the GenBank. The isolates selected for the
179 phylogenetic tree were representative samples of the antibiotic producing actinomycetes
180 from this study. A scrutiny of the 16S rRNA phylogenetic trees showed that isolate NWU195
181 formed a distinct phyletic line.

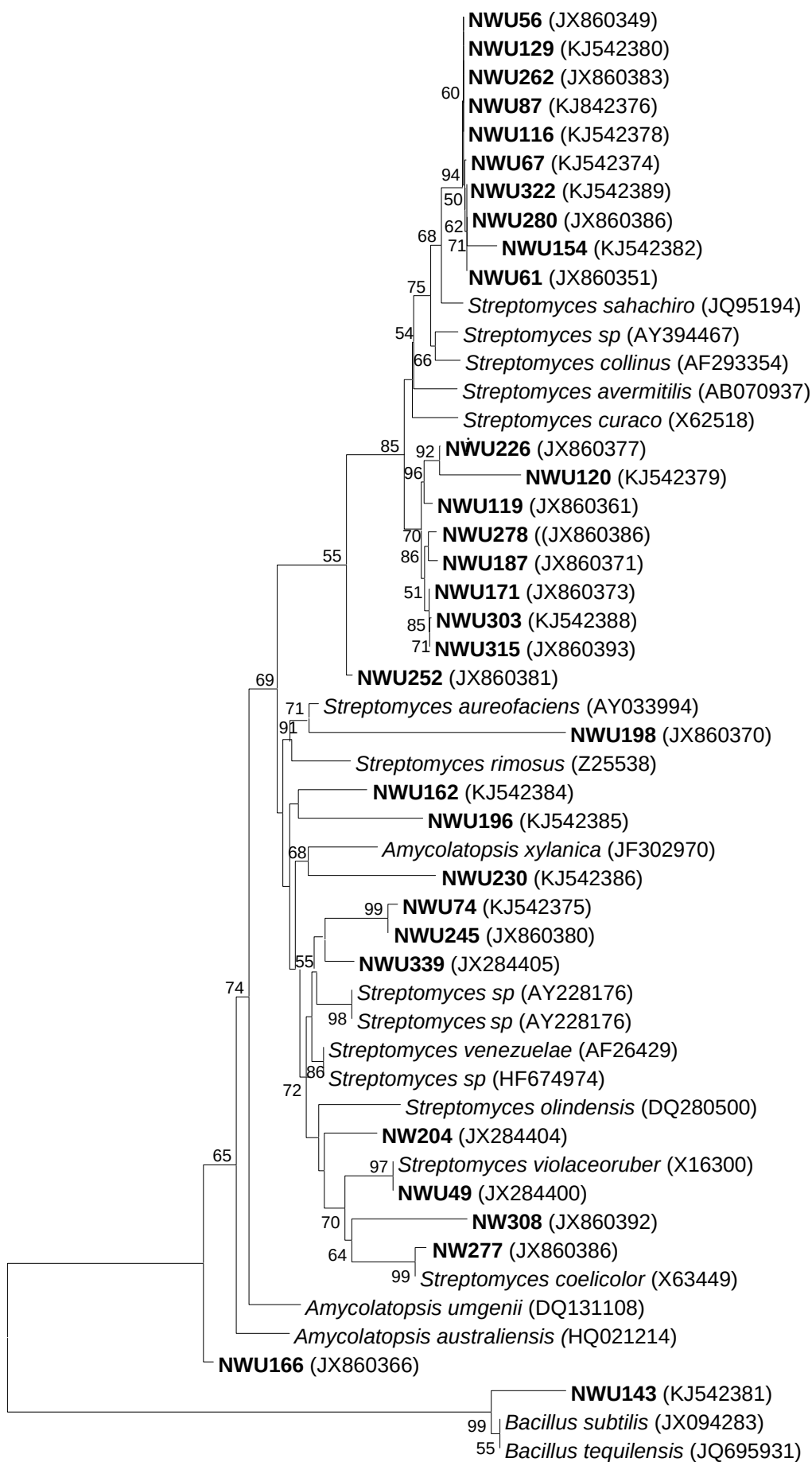
182 Further phylogenetic analysis was carried out on the positive strains for the biosynthetic
183 gene cluster with similarity to known antibiotic producing actinomycetes. The neighbor-
184 joining phylogenetic tree was constructed using the nucleotide sequences of AHSA synthase
185 gene obtained in this study and the gene segment from other PKS-I producers reference
186 sequences from the GenBank (Fig. 5). The nucleotide sequences of the AHSA synthase
187 gene from the positive strains previously identified as *Streptomyces* isolates formed a
188 distinct clade from other known antibiotic producer of the genus *Streptomyces*. The
189 *Micromonospora* spp cluster with NWU337 forming a phylogenetically distinct lineage.
190 Figure 6 shows the phylogenetic position of the isolates with known aromatic polyketide
191 producers based on the analysis of the PKS-II gene nucleotide sequences. The nucleotide
192 sequences of NRPS gene from the positive isolates and selected ones from the GenBank
193 were used in constructing the phylogenetic tree (Fig. 7). Clustering with other known
194 producers is evident. The lineage relationships of branches composed of the individual
195 isolates are supported by bootstrap values that are based on neighbor-joining analyses.



197 **Figure 4: Neighbour-joining tree (Saitou and Nei, 1987) based on 16S rRNA gene**
 198 **sequences showing relationships between actinomycetes strains and representatives**
 199 **of related taxa. Bootstrap value (>50%) based on 1000 resampled datasets are shown**
 200 **at branch nodes. *Bacillus subtilis* and *Bacillus tequilensis* are used as outgroup**

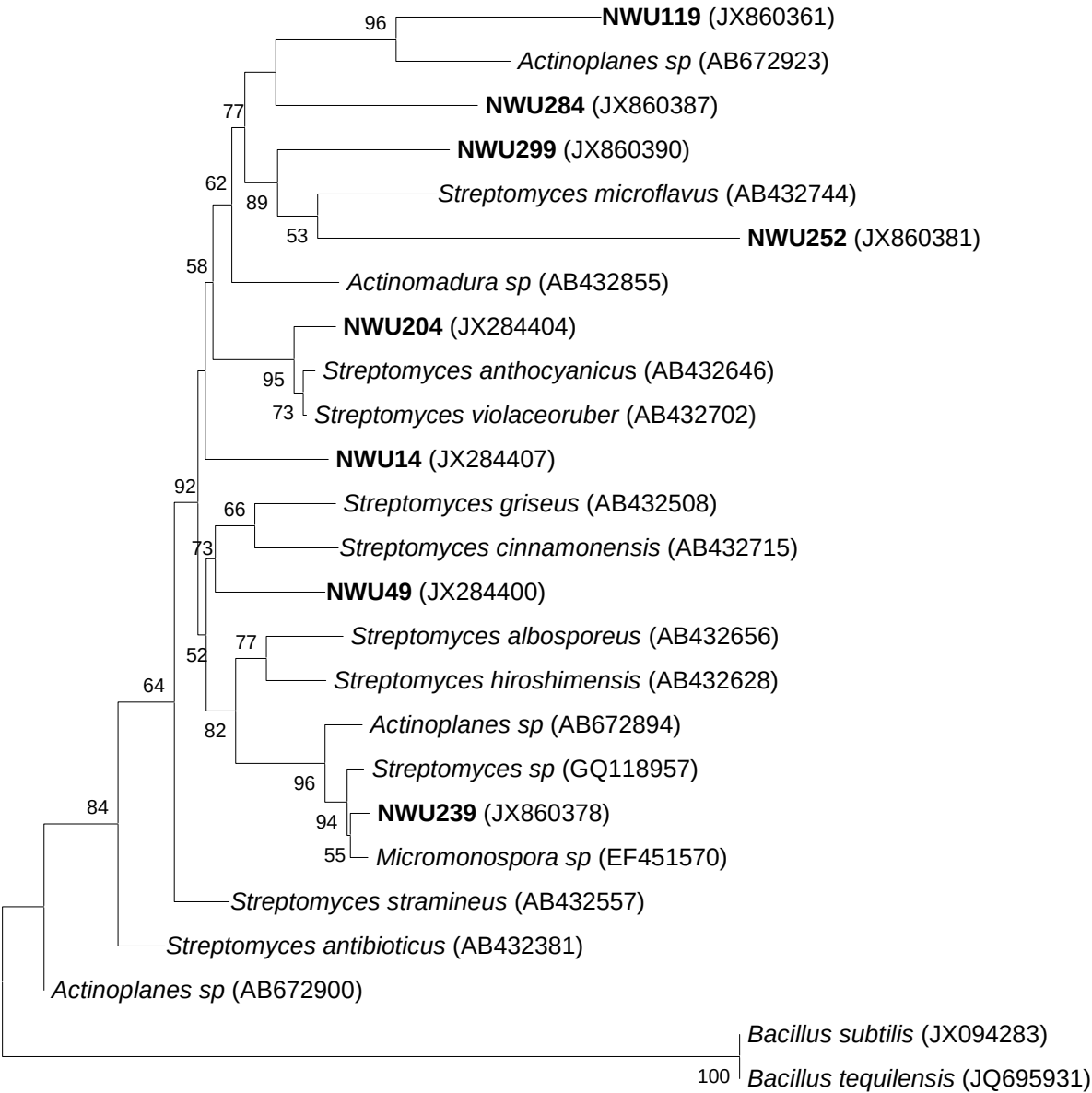


201
 202 **Figure 5: Neighbour-joining tree (Saitou and Nei, 1987) based on AHSA gene**
 203 **sequences showing relationships between actinomycetes strains and representatives**
 204 **of related taxa. Bootstrap value (>50%) based on 1000 resampled datasets are shown**
 205 **at branch nodes. *Bacillus subtilis* and *Bacillus tequilensis* are used as outgroup**



207
208
209
210
211
212
213
214
215
216
217
218

Figure 6: Neighbour-joining tree (Saitou and Nei, 1987) based on PKS-II gene sequences showing relationships between actinomycetes strains and representatives of related taxa. Bootstrap value (>50%) based on 1000 resampled datasets are shown at branch nodes. *Bacillus subtilis* and *Bacillus tequilensis* are used as outgroup



219

220 **Figure 7 Neighbour-joining tree (Saitou and Nei, 1987) based on NRPS gene**
221 **sequences showing relationships between actinomycetes strains and representatives**
222 **of related taxa. Bootstrap value (>50%) based on 1000 resampled datasets are shown**
223 **at branch nodes. *Bacillus subtilis* and *Bacillus tequilensis* are used as outgroup**

224 4. DISCUSSION

225 As part of bioprospecting programme, actinomycete isolates from rhizospheric soil samples
226 were screened in order to assess their antimicrobial potential, using degenerate primers to
227 detect the presence of PKS-I, PKS-II and NRPS genes. The PCR-based approach offers a
228 method by which to quickly screen a large collection of isolates in order to identify strains
229 with the potential to produce specific structural classes of secondary metabolites [2]. The
230 potential of actinomycetes to produce bioactive compounds has been exploited for years.
231 Recent genomic sequence analyses of the biosynthetic gene clusters have revealed a
232 previously unrecognized biosynthetic potential and diversity in actinomycetes [12].

233 The PCR-based approach can only indicate when a gene is present or absent through the
234 amplification of the gene or not. Some isolates such as NWU (14, 49, 119, 204, 220 and
235 339) exhibited antibacterial activity against selected test organisms and these correlated well
236 with successful amplification of either one or more of the targeted genes from the genomes
237 of the isolates. The PCR-based approach helps to expedite the screening of a large
238 collection of isolates in order to identify strains with the potential to produce bioactive
239 secondary metabolites [2, 20, 21]. It can be inferred that these isolates contain at least one
240 complete biosynthetic gene cluster for bioactive secondary metabolite production. Some
241 actinomycete isolates such as NWU (73, 91, 110, 208, 252, 284 and 336) exhibited
242 antimicrobial activity but there were no amplification products. The absence of PCR
243 amplicons in some of the isolates suggests the lack of the biosynthetic genes although it
244 cannot be concluded that the isolates in question lack such a gene. While it is possible that
245 the isolates do not harbor any PKS or NRPS genes, this might also be as a result of the less
246 conserved domain sequences sharing low homology with the primers in strains not well-
247 known to produce secondary metabolites of pharmaceutical importance [20]. Also according
248 to Wood et al. [3] this might be as result of variations in primer target sequences preventing
249 the primers from binding efficiently. A limitation in this kind of study is that the design of
250 degenerative primers is based on the available sequences in the GenBank of more
251 abundant and well-studied organisms such as *Streptomyces*, unlike the rare actinomycetes
252 that have few available sequences on their biosynthetic gene clusters in database library. As
253 the number of whole genome sequences for rare actinomycetes increases, this will help in
254 designing primers to amplify diverse biosynthetic genes. Some isolates such as NWU (87,
255 154, 196, 229, 267 and 322) also did not exhibit activity but there is amplification of the
256 biosynthetic gene cluster. According to Finking and Marahiel [17] not all biosynthetic gene
257 clusters are involved in the biosynthesis of bioactive secondary metabolites. It is also
258 possible that the genes detected by the PCR are non-functional or the isolates in question
259 might have different nutritional requirements for the production of bioactive secondary
260 metabolites [21, 22]. Also Wood et al. [3] concluded that positive PCR amplification does not
261 indicate that the genes are expressed nor does it show that the strain possesses the full
262 suite of biosynthetic gene cluster for the biosynthesis of that class of antibiotic. The detection
263 of PKS-I, PKS-II and NRPS genes in some isolates such as NWU (119, 204, 230 and 299)
264 are indicators of their potential natural product diversity and divergent genetic evolution.
265 Isolates with amplification of at least 2 of the primers used but with no antimicrobial activity
266 will be induced for the production of bioactive secondary metabolites. This might lead to the
267 discovery of novel compounds especially among the rare actinomycetes.

268 A phylogenetic approach using the nucleotide sequences generated from the DNA fragment
269 of the biosynthetic gene clusters (PKS-I, PKS-II and NRPS) of the positive actinomycete
270 isolates elucidated how nucleotide sequence information can be used to predict the number
271 and novelty of possible bioactive compounds through the phylogenetic relatedness to known
272 antibiotic producers. Phylogenetic analysis showed that most isolates have high bootstrap
273 values with known antibiotic producers. The study correlates with the finding of other
274 researchers [23-25] who isolated and screened for biosynthetic genes from actinomycetes.

275 As seen in Fig. 5, the isolates formed 2 distinct clades from other organisms on the
276 phylogenetic tree; this indicates the possibility of novel bioactive compounds biosynthesis
277 from the isolates. Although NWU337 is identified as *Streptomyces* sp, it clustered with
278 *Micromonospora* spp which are known producers of aminoglycoside antibiotics. In Fig. 6,
279 NWU198 clustered with *S. aureofaciens* and *S. rimosus* with bootstrap value of 91, these
280 organisms are known producers of tetracycline [7, 26]. Isolates NWU (49, 204, 277 and 308)
281 clustered with *S. olindensis*, *S. violaceoruber* and *S. coelicolor*, known producers of
282 antibiotics [27]. NWU277 has a bootstrap value of 99 with *S. coelicolor*, a prolific producer of
283 antibiotics such as actinorhodin, methylenomycin, undecylprodigiosin, and perimycin [7, 28]
284 in Fig. 7, isolate NWU14 formed a distinct phyletic line suggesting it might produce novel
285 bioactive compound. Isolate NWU49 clustered together with *S. griseus* and *S.*
286 *cinnamomensis* with a bootstrap value of 77, showing that it is close relative to these
287 organisms which are known producers of streptomycin and monensin respectively [29, 30].
288 Isolates in close phylogenetic relationships with known antibiotic producers might also be
289 involved in the biosynthesis of the same or similar bioactive compounds since they share
290 similar features [31].

291 5. CONCLUSION

292 The PCR-based approach helps identify actinomycete isolates with biosynthetic gene
293 clusters for the production of bioactive secondary metabolites. The presence of a particular
294 gene gives an indication of the type of biosynthetic pathway to be explored. From the result
295 generated in this study, there is a clear relationship between the occurrence of biosynthetic
296 gene clusters and production of antimicrobial agents. The advantage of the PCR-based
297 approach for novel drugs is that it provides knowledge about the type of prospective
298 compounds that can be produced by the strain having the biosynthetic gene. While the
299 information is expedient, phylogenetic analysis of biosynthetic genes associated with the
300 production of secondary metabolites can give an insight into both the novelty and diversity of
301 pathways present within an isolate. All these give an insight about the applicable extraction
302 and purification technique to be applied for that class of bioactive compound. Fermentation
303 extracts from some of the actinomycete isolates are currently being analyzed chemically in
304 order to identify the bioactive compounds and assess their novelty.

305 REFERENCES

- 308 1. Wenzel SC, Müller R. Recent developments towards the heterologous expression of
309 complex bacterial natural product biosynthetic pathways. Curr Opin Biotechnol.
310 2005;16(6):594-606.
- 311 2. Gontang EA, Gaudêncio SP, Fenical W, Jensen PR. Sequence-based analysis of
312 secondary-metabolite biosynthesis in marine actinobacteria. Appl Environ Microbiol.
313 2010;76(8):2487-99.
- 314 3. Wood SA, Kirby BM, Goodwin CM, Le Roes M, Meyers PR. PCR screening reveals
315 unexpected antibiotic biosynthetic potential in *Amycolatopsis* sp. strain UM16. J Appl
316 Microbiol. 2007;102(1):245-53.

- 317 4. Gontang EA. Phylogenetic diversity of gram-positive bacteria and their secondary
318 metabolite genes: ProQuest; 2008.
- 319 5. Berdy J. Bioactive microbial metabolites. J Antibiot. 2005;58(1):1-26.
- 320 6. Ichikawa N, Sasagawa M, Yamamoto M, Komaki H, Yoshida Y, Yamazaki S, et al.
321 DoBISCUIT: a database of secondary metabolite biosynthetic gene clusters. Nucleic Acids
322 Res. 2013;41(D1):D408-D14.
- 323 7. Adegboye M, Babalola O. Actinomycetes: a yet inexhaustive source of bioactive
324 secondary metabolites. In: Microbial pathogens and strategies for combating them: science,
325 technology and education. A. Méndez Vilas, ed. Formatex Research Center, Badajoz, Spain.
326 2013.
- 327 8. Adegboye MF, Babalola OO. Isolation, characterization and antibacterial activity of
328 Streptomycetes from rhizosphere soils in North West Province, South Africa. Asia Life Sci.
329 2013;9(2):403-21.
- 330 9. Zin N, Tasrip NA, Desa M, Kqueen CY, Zakaria ZA, Hamat RA, et al. Characterization
331 and antimicrobial activities of two Streptomyces isolates from soil in the periphery of
332 Universiti Putra Malaysia. Trop Biomed. 2011;28:651-60.
- 333 10. Manfioletti G, Schneider C. A new and fast method for preparing high quality lambda
334 DNA suitable for sequencing. Nucleic Acids Res. 1988;16(7):2873-84.
- 335 11. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for
336 phylogenetic study. J Bacteriol. 1991;173(2):697-703.
- 337 12. Ayuso-Sacido A, Genilloud O. New PCR primers for the screening of NRPS and PKS-
338 I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences
339 in major taxonomic groups. Microb Ecol. 2005;49(1):10-24.
- 340 13. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
341 tool. J Mol Biol. 1990;215(3):403-10.
- 342 14. Katoh S. MAFFT multiple sequence alignment software version 7: improvements in
343 performance and usability. Mol Biol Evol. 2013;30:772-80.
- 344 15. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular
345 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and
346 maximum parsimony methods. Mol Biol Evol. 2011;28: 2731-9.
- 347 16. Saitou N, Nei M. The Neighbor-Joining Method—a new method for reconstructing
348 phylogenetic trees. Mol Biol Evol. 1987;4:406–25.
- 349 17. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap.
350 Evol. 1985;39:783–91.
- 351 18. Page RDM. TREEVIEW: an application to display phylogenetic trees on personal
352 computers. Comput Appl Biosci. 1996;12:357-8.
- 353 19. Adegboye MF, Babalola OO. Phylogenetic characterization of culturable antibiotic
354 producing *Streptomyces* from rhizospheric soils. Mol Biol. 2013;1:2.
- 355 20. Lee L-H, Zainal N, Azman A-S, Eng S-K, Goh B-H, Yin W-F, et al. Diversity and
356 Antimicrobial Activities of Actinobacteria Isolated from Tropical Mangrove Sediments in
357 Malaysia. Scientific World J. 2014;2014.
- 358 21. Qin S, Li J, Chen H-H, Zhao G-Z, Zhu W-Y, Jiang C-L, et al. Isolation, diversity, and
359 antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in
360 Xishuangbanna, China. Appl Environ Microbiol. 2009;75(19):6176-86.
- 361 22. Janso JE, Carter GT. Biosynthetic potential of phylogenetically unique endophytic
362 actinomycetes from tropical plants. Applied Environ Microbiol. 2010;76(13):4377-86.
- 363 23. Bredholdt H, Galatenko OA, Engelhardt K, Fjaervik E, Terekhove LP, Johsen G, et al.
364 Rare actinomycete bacteria from the shallow water sediments in Trondheim Fjord Norway:
365 Isolation, diversity and biological activity. Environ Microbiol. 2007;9:2756-64.
- 366 24. Bai Y, Yang D, Wang J, Xu S, Wang X, An L. Phylogenetic diversity of culturable
367 bacteria from alpine permafrost in the Tianshan Mountains, Northwestern China. Res
368 Microbiol. 2006;157(8):741-51.

- 369 25. Hwang Y-B, Lee M-Y, Park H-J, Han K, Kim E-S. Isolation of putative polyene-
370 producing actinomycetes strains via PCR-based genome screening for polyene-specific
371 hydroxylase genes. *Process Biochem.* 2007;42(1):102-7.
- 372 26. Arias AA, Craig M, Fickers P. Science against microbial pathogens: *In* communicating
373 current research and technological advances. Badajoz, Spain: Formatex Research Center;
374 2011.
- 375 27. de Lima Procópio RE, da Silva IR, Martins MK, de Azevedo JL, de Araújo JM.
376 Antibiotics produced by *Streptomyces*. *Braz J Infect Dis.* 2012;16(5):466-71.
- 377 28. Wang G, Hosaka T, Ochi K. Dramatic activation of antibiotic production in
378 *Streptomyces coelicolor* by cumulative drug resistance mutations. *Appl Environ Microbiol.*
379 2008;74(9):2834-40.
- 380 29. Li C, Florova G, Akopiants K, Reynolds KA. Crotonyl-coenzyme A reductase provides
381 methylmalonyl-CoA precursors for monensin biosynthesis by *Streptomyces cinnamonensis*
382 in an oil-based extended fermentation. *Microbiol.* 2004;150(10):3463-72.
- 383 30. Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, et al. Genome
384 sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J*
385 *Bacteriol.* 2008;190(11):4050-60.
- 386 31. Wang HX, Chen YY, Ge L, Fang TT, Meng J, Liu Z, et al. PCR screening reveals
387 considerable unexploited biosynthetic potential of ansamycins and a mysterious family of
388 AHBA-containing natural products in actinomycetes. *J Appl Microbiol.* 2013;115(1):77-85.
389