Food Microbiology, Volume 116, December 2023, Article number 104370
DOI:10.1016/j.fm.2023.104370

Short communication

SRAP markers as an alternative tool for *Alternaria* classification

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Abstract

Alternaria is one of the main fungal contaminants of cereal grains worldwide with the potential to produce mycotoxins hazardous to human and animal health. Many studies have been carried out to characterize *Alternaria* sp.-grp. using traditional morphology or polyphasic approach, but a good correlation between morphological sp.-grp., molecular, and chemotaxonomic groups has not always been achieved. For this reason, this study aimed to investigate the usefulness of a cheaper alternative tool, SRAP markers, in identifying *Alternaria* sp.-grps. obtained from Argentinean barley grains and to compare it with preliminary characterization using morphological traits, phylogeny, and metabolite profiles. Fifty-three *Alternaria* isolates from barley grains of the main producing regions of Argentina were analyzed with four combinations of SRAP markers. The UPGMA dendrogram, based on the Simple Matching similarity coefficient, revealed three distinct groups. SRAP markers allowed the separation of *Alternaria* from *Infectoriae* sections in agreement with the results of a polyphasic approach previously made. Besides, isolates of *A. arborescens* sp.-grp. were clustered in a separate group from isolates of *A. tenuissima* and *A. alternata* sp.-grp., which were grouped in the same cluster. SRAP markers are a recommended tool for classifying *Alternaria* isolates because of its simplicity, reliability, and cost-effectiveness compared to other molecular markers.

Keywords: *Alternaria*, characterization, molecular marker, SRAP

1. Introduction

Alternaria is one of the main fungal contaminants of cereal grains worldwide, with incidences up to 60% in favourable seasons, causing yield losses and affecting the quality of the infected crop products (Müller et al., 2013; Kulik et al., 2015; Barkat et al., 2016; Krasauskas, 2017; Beccari et al., 2018; Castañares et al., 2021). Besides, some *Alternaria* species have the potential to produce a high range of mycotoxins, which have drawn increasing attention in recent years because of its genotoxic, mutagenic, and carcinogenic properties and its frequent occurrence in several cereals and by-products (Zwickel et al., 2018; Braun et al., 2020; Babič et al., 2021; Orina et al., 2021; Scheibenzuber et al., 2021; Prusova et al., 2022). The small spored *Alternaria* species (spore size < 60 µm) are the most relevant in cereal grains, mainly those belonging to sections *Alternaria* and *Infectoriae*. *Alternaria* section *Alternaria*, includes *A. alternata* species-group, *A. tenuissima* sp.-grp. and *A. arborescens* sp.-grp.. These species are able to produce some of the most important and frequent mycotoxins, such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), tentoxin (TEN), and altertoxins (ATXs). Whereas isolates belonging to *Alternaria* section *Infectoriae*, with *A. infectoria* as the main representative sp.-grp., are characterized by a different secondary metabolite profile, including infectopyrones, novae-zelandins, phomapyrones, and altertoxin-like metabolites, like alterperylenol, whose toxicity has not yet been thoroughly investigated (Andersen et al., 2015; Lawrence et al., 2016; Tralamazza et al., 2018).

Many efforts have been carried out to characterize *Alternaria* sp.-grp. based on traditional morphological identification alone (Simmons, 2007) or in combination with phylogenetic studies (Andrew et al., 2009; Woudenberg et al., 2013; Lawrence et al., 2013; Woudenberg et al., 2015) or metabolite profiles (Andersen et al., 2015; Patriarca et al., 2019). Besides, many studies combined these tools in a polyphasic approach to achieve more accurate identification (Kahl et al., 2015; da Cruz Cabral et al., 2017; Siciliano et al., 2018; Castañares et al., 2021), although not always reaching a strict correlation between morphological sp.-grps., chemotaxonomic and molecular groups. In Argentina, a study carried out with a polyphasic approach identified small-spored species belonging to *Alternaria* section *Alternaria* (*A. tenuissima*, *A. alternata*, *A. arborescens* sp.-grps.) and *Alternaria* section *Infectoriae* (*A. infectoria* sp.-grps. and *A. vaccinii*) from barley grains with a distinctive metabolite profile (Castañares et al., 2021). Correctly identifying the species of *Alternaria* genus is important to predict the metabolite profiles that can be expected as contaminants of a particular crop. On the other hand, DNA fingerprinting, such as

amplified length polymorphism (AFLP), random amplified polymorphism DNA (RAPD), and inter simple-sequence repeats (ISSR) markers, have also been used to study genetic diversity and relationships among different *Alternaria* sp.-grps. from diverse crops. In some cases, a separation among sections and even morphological sp.-grps. was obtained (Morris et al., 2000; Bock et al., 2002; Hong et al., 2006; Luo et al., 2017; Zelmat et al., 2021). However, since these molecular markers are complex, expensive, and do not always provide consistent results, the use of a fast, easy to perform and cheap technique is necessary to identify this genus. Sequence-Related Amplified Polymorphism (SRAP) is a PCR-based assay developed to target open reading frames (ORFs) that encode proteins of interest and requires no knowledge of nucleotide sequence. Because of its simplicity and inexpensiveness compared to other molecular methods, it has been used to study genetic diversity and population structure of fungi (Guo et al., 2008; Pasquali et. al., 2010; Ren et al., 2012; Dinolfo et al., 2015; Castañares et al., 2016).

This work aimed to investigate the efficacy of SRAP markers in identifying small-spored *Alternaria* species obtained from Argentinean barley grains and to compare it with a preliminary characterization based on morphological traits, phylogeny, and metabolite profiles. These results could offer an additional tool for the classification of food-borne *Alternaria* sp.-grps.

2. Materials and Methods

2.1. Fungal isolates

Fifty-three *Alternaria* isolates from barley grains of the main producing regions of Argentina were included in the analysis. Monosporic cultures of the isolates were previously characterized through a polyphasic approach by Castañares et al. (2021) as belonging to four *Alternaria* sp.-grps. (*A. tenuissima*, *A. infectoria*, *A. arborescens*, and *A. alternata*) and one was identified to species level as *A. vaccinii*. Besides, four representative *Alternaria* strains of the main sp.-grps. (EGS collection) and five *Alternaria* isolates from oat grains were also included for comparison (Table 1). *Alternaria* isolates from oat grains of the main producing region of Buenos Aires province were morphologically identified from monosporic cultures grown in PCA, according to Simmons, (2007) (data not published). Genomic DNA extraction of the sixty-two isolates was made using CTAB (cetyltrimethylammonium Bromide) method, according to Stenglein & Ballati, (2006). DNA quality was examined by electrophoresis in 0.8% agarose gels with

GelRed™ (Biotium, Hayward, USA) at 80 V in 1 X Trisborate-EDTA buffer and visualized under UV light. In contrast, DNA concentration was calculated by fluorometry (Qubit™ Invitrogen, Argentina). Extracted DNA was stored at -20 °C until analysis.

2.2. SRAP markers

A subset of four out of nine combinations of SRAP markers was selected based on the production of polymorphic fragments and reproducible banding patterns (ME5/EM12; ME1/EM12; ME5/EM17; ME6/EM8). The sequences of each primer are shown in Table 2. PCR reactions were performed in XP Thermal cycler (Bioer Technology Co., China) with a final volume of 25 µL containing: 10-20 ng of genomic DNA, 10 X reaction buffer, 25 mM $MgCl_2$, 5 mM dNTP, 50 µM of each primer (forward and reverse), and 5000 U/mL of Taq DNA polymerase (Inbio-Highway, Argentina). PCR conditions were as described by Li & Quiros (2001): 95 °C for 5 min, 94 °C for 1 min, 35 °C for 1 min, 72 °C for 1 min for 5 cycles. The annealing temperature was then increased to 50 °C for the subsequent 35 cycles. Finally, an extension at 72 °C for 10 min was made. Each reaction was made at least twice. Amplified fragments were separated by vertical electrophoresis in polyacrylamide gels at 54 mA for 4 h and then revealed by silver staining, according to Bassam et al., (1991). All generated fragments were analyzed.

2.3. Data analyses

Amplified fragments were manually scored in a binary matrix in which "1" represent presence and "0" absence of each fragment. Unresolved data and missing data were treated as missing data. The genetic distance between isolates was estimated based on the Simple Matching similarity coefficient. The cophenetic correlation coefficient (CCC) was used to indicate the level of distortion between the similarity matrix and cluster analysis. Dendrograms were constructed using the UPGMA algorithm in the statistical software NTSYSPC v. 2.0 (Rohlf, 1998).

3. Results

The analysis of 62 isolates, including *Alternaria* isolates from barley and oat grains and reference strains of EGS collection, generated a binary matrix in which 76 fragments were analyzed. The number of fragments generated by each primer combination varied between 17 (ME5/EM17) and 21 (ME5/EM12). The primer set ME6/EM8 gave a total of 19 fragment amplifications, of which only two were monomorphic, indicating 89,5% DNA polymorphism among the genotypes. The other primer

combinations showed 100% of DNA polymorphism. Only two *A. arborescens* sp.-grp. isolates had the same haplotype. The UPGMA dendrogram, based upon the Simple Matching similarity coefficient calculated from the combined dataset, is shown in Fig. 1. Three distinct groups were distinguished. Group A contained all isolates belonging to *A. tenuissima* sp.-grp., the three isolates belonging to *A. alternata* sp.-grp., and the reference strains of these sp.-grps. (EGS 34016 and EGS 34015). Group B contained all isolates belonging to *A. arborescens* sp.-grp. and their reference strain EGS 39128. Isolates of *A. infectoria* sp.-grp., their reference strain EGS27198, and the two *A. vaccinii* isolates were clustered in the third group (group C) at a similarity value of about 56% concerning the other two groups. In turn, the *Alternaria* isolates from oat grains were distributed in groups A or C, in accordance with their morphological identification, together with the barley isolates. Besides, specific groups based on the geographical origin of the isolates were not obtained.

4. Discussion

Taxonomic *Alternaria* characterization is highly complex, especially among the small-spore sp.-grps., because of the overlapping morphological characteristics, low variability among DNA sequences and similar metabolite profile within the same sections. Their distinction is a challenge since many *Alternaria* spp. act as phytopathogens causing different plant diseases, for instance, *A. mali* is considered an organism of quarantine importance, causing Alternaria bloth of apple, *A. alternata* causes leaf spot in several host plants, and *A. arborescens* damages tomato and apple (Rotem 1994; Johnson et al., 2000; Rotondo et al., 2012; Harteveld et al., 2013). Moreover, as mentioned before, the metabolite profile effectively separates isolates of *Alternaria* and *Infectoriae* sections, while it seems similar among the sp.-gps. of *Alternaria* section. However, many studies suggest a distinctive metabolite profile among sp.-grps. of *Alternaria* section, which could be a tool to determine the risk in animal and human health (Andersen et al. 2001, 2002; Polizzoto et al. 2012; Siciliano et al. 2018). Furthermore, the correct identification of *Alternaria* sp.-grps. is needed to evaluate the prevalence of a pathogen in a particular host and thus assess changes in the fungal communities at different geographical sites or under different climatic conditions, which is an important tool to establish control strategies. For this reason, the present study aimed to provide an additional tool for classifying food-borne *Alternaria* spp. The *Alternaria* species commonly infecting cereal grains belong to *Alternaria* and *Infectoriae* sections. In a previous study carried out in barley grains, Castañares et al., (2021) showed the following composition of the

Alternaria population of malting barley: 73% of the isolates morphologically identified as belonging to *A. tenuissima* sp.-grp., 14.6% to the *A. infectoria* sp.-grp., 5% to the *A. arborescens* sp.-grp., and 3.9% to the *A alternata* sp.-grp.; 1,4% were identified to species level as *A. vaccinii*, while 2.1% of the isolates showed intermediate characteristics and were referred to as *Alternaria* spp. Although metabolite profile and phylogenetic analysis allowed the separation of sections *Alternaria* and *Infectoriae*, it was difficult to distinguish among sp.-grps. within the *Alternaria* section. Only a partial separation was achieved through sequencing the OPA 10-2 region among sp.-grps. of the *Alternaria* section (Castañares et al., 2021). Many researchers have used DNA fingerprinting in an attempt to study genetic diversity and solve *Aternaria* characterization. Somma et al., (2011), in a study carried out using AFLP markers, revealed two main clusters with *A. tenuissima* and *A. alternata* sp.-grps. in one of them and *A. arborecens* sp.-grp. in the other. Similar results using AFLP markers were obtained by Hong et al., (2006) and Luo et al., (2017). However, in the same study, Hong et al., (2006) could not resolve *A. alternata* from *A. arborescens* sp.-grps. with ISSR markers. Likewise, AFLP was a reliable tool to identify isolates of *A. brassicicola* (Bock et al., 2002). Nevertheless, AFLP markers are relatively costly and time-consuming compared to other marker systems such as SRAP, which combines simplicity and reliability, and is inexpensive and a very informative method. The use of SRAP markers in the present study allowed the separation between *Infectoriae* and *Alternaria* sections, which is in agreement with the polyphasic approach mentioned before. Even more, within the *Alternaria* section, and in agreement with the studies with AFLP markers, two groups were segregated with *A. tenussima* sp.-grp. and *A. alternata* sp.-grp. in one of them and *A. arborescens* sp.-grp. in the other. Despite the distinct morphological pattern of *A. tenuissima* and *A. alternata* sp.-grps. described by Simmons, (2007), the present study, as well as others (Hong et al., 2006; Somma et al., 2011; Woudenberg et al., 2015; Luo et al., 2017), failed to resolve these sp.-grps. using molecular methods. For this reason, many authors suggest considering them as a simple taxon until more information is obtained (Andrew et al., 2009; Somma et al., 2011; Woudenberg et al., 2015; Luo et al., 2017). SRAP markers were also used in the study of Zelmat et al., (2021) to evaluate the genetic diversity and population structure of *Alternaria* section *Alternaria* isolated from infected citrus fruits. The results showed a cluster with four groups but a separation among sp.-grps. of the same section was not achieved. Besides, in the same work, a phylogenetic analysis based on ITS sequences was made clustering in the same group reference strains of *A. tenuissima*, *A. alternata*, *A. arborescens*, *A. gaisen,* and *A. longipes*. Concerning the absence of grouping of the *Alternaria* isolates by geographical origin

using SRAP markers, our results agree with those from Khal et al. (2015), who found a slight difference in sp.-grps. composition among Rusian and German regions as was observed in the different localities sampled from Argentina by Castañares et al. (2021), but not regional-specific clusters based on mycotoxins and phylogenetic assays.

On the other hand, SRAP markers have been used for fungal classification yielding good results in several studies. For example, Ren et al., (2012) suggested the use of SRAP markers to identify endophytic fungi from Taxus. Besides, Castañares et al., (2016) showed that SRAP markers allowed to separate *F. graminearum sensu stricto* from the rest of the *Fusarium graminearum* species complex, while AFLP markers failed to resolve them into distinctive groups. According to our results, SRAP markers are a recommended alternative tool for classifying *Alternaria* isolates because it is a simple, reliable and cost-effective PCR-based assay compared to other molecular markers.

Acknowledgments The authors sincerely thanks Daiana Fernandez and Germán Pacheco for their technical assistance. This research was supported by Agencia Nacional de Promoción Científica y Tecnológica-FONCyT [PICT 2055/2017 and PICT 2020/0731] and UNCPBA.

Declarations of interest none

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308

309 T**ables**

310 Table 1. *Alternaria* isolates from barley and oat grains used in the study.

Strain name	***Alternaria* sp.-gp.**	**Host**	**Year**	**GenBank Accession number***
Alt16/1b	*A. alternata* sp.-grp.	Barley	2014	----------
Alt6/19	*A. alternata* sp.-grp.	Barley	2014	----------
Alt25/4	*A. alternata* sp.-grp.	Barley	2014	MT977660 MT977637
Alt44/a7	*A. arborescens* sp.-grp.	Barley	2015	----------
Alt46/1	*A. arborescens* sp.-grp.	Barley	2015	----------
Alt33/8	*A. arborescens* sp.-grp.	Barley	2015	----------
Arb2/5	*A. arborescens* sp.-grp.	Barley	2014	----------
Arb13/15	*A. arborescens* sp.-grp.	Barley	2014	----------
Arb14/1	*A. arborescens* sp.-grp.	Barley	2014	----------
Arb4/1	*A. arborescens* sp.-grp.	Barley	2014	MT977657 MT977634
Arb38/a16	*A. arborescens* sp.-grp.	Barley	2015	----------
Arb41/a3	*A. arborescens* sp.-grp.	Barley	2015	----------
Arb61/1c	*A. arborescens* sp.-grp.	Barley	2015	----------
Arb36/a1	*A. arborescens* sp.-grp.	Barley	2015	MT977662 MT977639
Arb64/37	*A. arborescens* sp.-grp.	Barley	2015	MT977665 MT977641
Ten8/9	*A. tenuissima* sp.-grp.	Barley	2014	----------
Ten10/10	*A. tenuissima* sp.-grp.	Barley	2014	----------
Ten1/5	*A. tenuissima* sp.-grp.	Barley	2014	----------
Ten4/3	*A. tenuissima* sp.-grp.	Barley	2014	----------
Ten14/10	*A. tenuissima* sp.-grp.	Barley	2014	----------
Ten32/6	*A. tenuissima* sp.-grp.	Barley	2014	MT977661 MT977638
Ten5/1b	*A. tenuissima* sp.-grp.	Barley	2014	MT977658 MT977635
Ten32/1d	*A. tenuissima* sp.-grp.	Barley	2014	----------
Ten42/1b	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten62/1e	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten61/29	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten48/1b	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten37/a3	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten33/7b	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten45/1	*A. tenuissima* sp.-grp.	Barley	2015	----------
Ten40/10	*A. tenuissima* sp.-grp.	Barley	2015	MT977663 MT977640
Ten44/a5	*A. tenuissima* sp.-grp.	Barley	2015	MT977651 MT977633
Inf26/7b	*A. infectoria* sp.-grp.	Barley	2014	----------
Inf28/1c	*A. infectoria* sp.-grp.	Barley	2014	----------
Inf4/8f	*A. infectoria* sp.-grp.	Barley	2014	----------
Inf16/6d	*A. infectoria* sp.-grp.	Barley	2014	----------
Inf2/3b	*A. infectoria* sp.-grp.	Barley	2014	MT977656
Inf60/1e	*A. infectoria* sp.-grp.	Barley	2015	----------
Inf64/4d	*A. infectoria* sp.-grp.	Barley	2015	----------
Inf48/1e	*A. infectoria* sp.-grp.	Barley	2015	----------
Inf35/a4	*A. infectoria* sp.-grp.	Barley	2015	MT977644
Inf36/h7	*A. infectoria* sp.-grp.	Barley	2015	MT977647
Inf36/i1	*A. infectoria* sp.-grp.	Barley	2015	----------
Inf36/f3	*A. infectoria* sp.-grp.	Barley	2015	----------
Inf58/2d	*A. infectoria* sp.-grp.	Barley	2015	MT977655
Inf58/1c	*A. infectoria* sp.-grp.	Barley	2015	MT977666
Inf36/i8	*A. infectoria* sp.-grp.	Barley	2015	MT977649
Inf44/b19	*A. infectoria* sp.-grp.	Barley	2015	MT977652
Inf58/8c	*A. infectoria* sp.-grp.	Barley	2015	MT977667
Inf36/h3	*A. infectoria* sp.-grp.	Barley	2015	MT977646
Inf36/h9	*A. infectoria* sp.-grp.	Barley	2015	MT977648
Vac57/1b	*A. vaccinii*	Barley	2015	MT977653
Vac60/1d	*A. vaccinii*	Barley	2015	MT977664
Ten17/1Av	*A. tenuissima* sp.-grp.	Oat	2018	----------

Ten17/2Av	*A. tenuissima* sp.-grp.	Oat	2018	----------
Ten17/3Av	*A. infectoria* sp.-grp.	Oat	2018	----------
Inf17/5Av	*A. infectoria* sp.-grp.	Oat	2018	----------
Inf30/1Av	*A. infectoria* sp.-grp.	Oat	2018	----------

311 *Accession number of sequences previously described by Castañares et al., (2021).

312

313 Table 2. Sequences of SRAP primers used in the study.

314

SRAP primer	Sequence (5´ - 3´)
ME 1	TGA GTC CAA ACC GGA TA
ME 5	TGA GTC CAA ACC GGA AG
ME 6	TGA GTC CAA ACC GGT AA
EM 8	GAC TGC GTA CGA ATT CTG
EM 12	GAC TGC GTA CGA ATT GTC
EM 17	GAC TGC GTA CGA ATT CCA

318

319

320 **Figure legend**

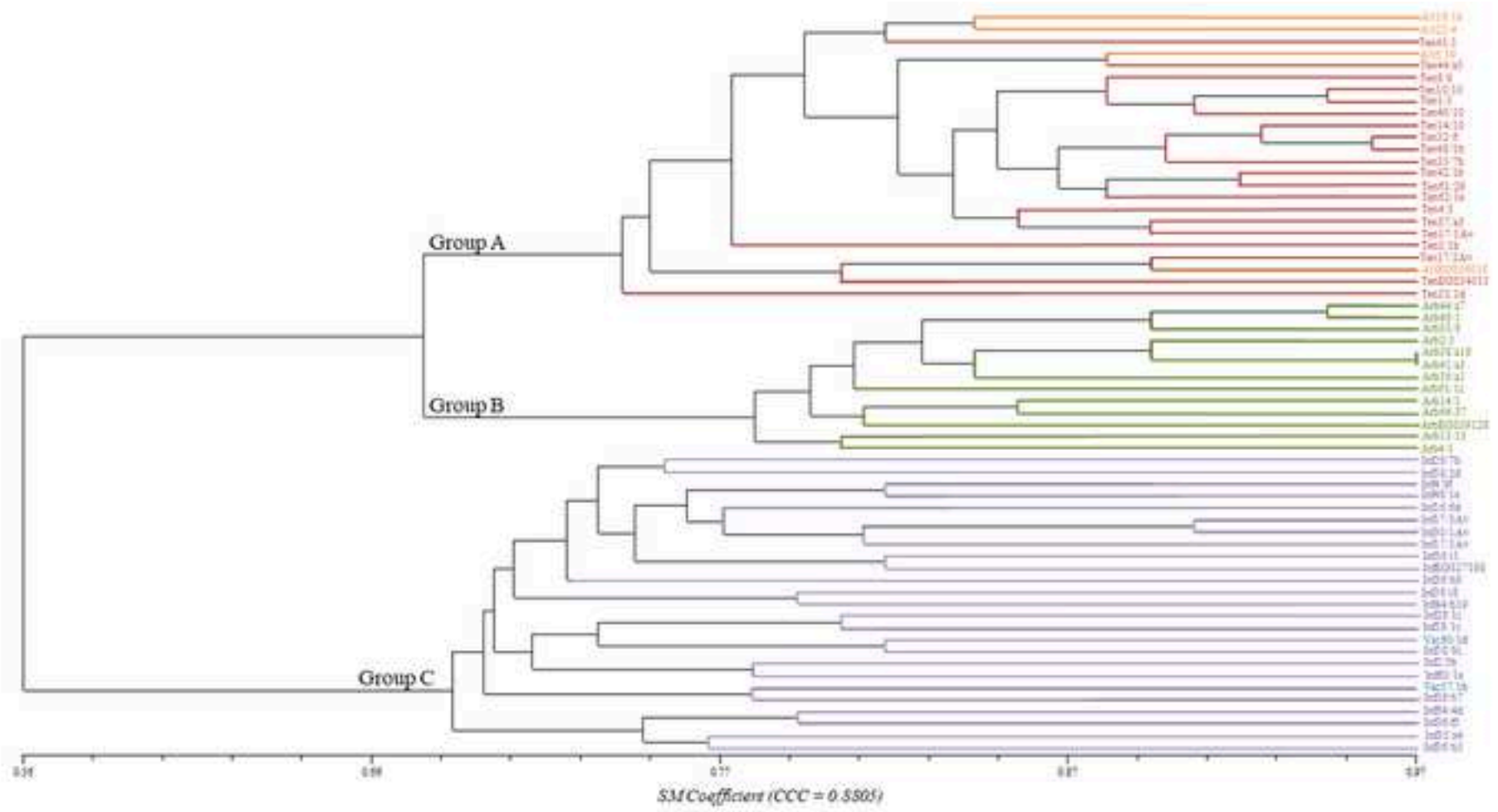
321 Figure 1. Dendrogram obtained from SRAP markers based upon the Simple Matching similarity
322 coefficient. Ten: *A. tenuissima* sp.-grps. (red); Alt: *A. alternata* sp.-grps. (orange); Arb: *A. arborescens*
323 sp.-grps. (green); Inf: *A. infectoria* sp.-grps. (violet); Vac: *A. vaccinii* (light blue).

324

325

Figure

Click here to access/download;Figure;Fig 1.png



SRAP markers as an alternative tool for Alternaria classification

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2023-08-28

Castañares E, Dinolfo MI, Patriarca A, Stenglein SA. (2023) SRAP markers as an alternative tool for Alternaria classificatio. Food Microbiology, Volume 116, December 2023, Article number 104370 https://doi.org/10.1016/j.fm.2023.104370