Lachancea lanzarotensis sp. nov., an ascomycetous yeast isolated from grapes and wine fermentation in Lanzarote, Canary Islands

Sara S. González,1 Julia Alcoba-Flórez2 and Federico Laich3

During the characterization of the microbiota biodiversity associated with grapes and wineries in different bioclimatic conditions of the Canary Islands (Spain), a novel yeast species was isolated from Lanzarote, the driest wine-producing region of the archipelago. Seven strains isolated from grapes, microvinifications and wineries are described. Sequence analysis of the D1/D2 domain of the LSU rDNA gene and 5.8S-ITS regions revealed that the isolates were phylogenetically a member of the genus Lachancea and are closely related to *Lachancea meyersii* NRRL Y-27269T and *Lachancea nothofagi* NRRL Y-48670T. On the basis of morphological, biochemical and physiological characterization and phylogenetic analysis, a novel ascosporogenous yeast species, *Lachancea lanzarotensis* sp. nov., is proposed. The type strain is L2C-15T (=CBS 12615T = CECT 13066T) which was isolated from grape berries of *Vitis vinifera* L. cv. Listán Negro red grape variety in Tinajo, Lanzarote. The MycoBank no. is MB 801390.

**Introduction**

Yeasts are part of the natural microbial communities associated with fresh grapes and are the most important micro-organisms in wine production (Fleet et al., 2002). The yeast community on grapes is influenced by biotic and abiotic factors, including climatic conditions (mainly temperature and rainfall), geographical location and vineyard factors (age, size, grape variety and vintage year), vineyard treatments, physical grape damage, microbial vectors, microbial interactions and enzymic activities (Barata et al., 2012). Spontaneous alcoholic fermentation of grape must is characterized by the presence of a high number of yeast genera and species. In grape must and during the early phase of wine fermentation, apiculate species are the most frequent yeasts. As fermentations continue and ethanol concentration increases, *Saccharomyces cerevisiae* and related species become the dominant yeasts and complete the process (Fleet & Heard, 1993). Indigenous yeast species from grapes and wine have been described in many wine-producing regions of the world.

The Canary Islands, located in the Atlantic Ocean, near the Tropic of Cancer, off the African coast of the Western Sahara, have a long tradition of viticulture. The Canarian archipelago comprises seven main islands together with a number of smaller islets. Most Canarian wines are produced in Lanzarote, Tenerife and La Palma. These three islands have different bioclimatic and topographic characteristics. The relief is the main factor that affects the local rainfall distribution. In general, precipitation increases across the archipelago from east to west. Tenerife is situated near the centre of the archipelago and has the largest number of bioclimatic belts (26). Maximum rainfall is recorded on the northern side (up to 500 mm per year), while the southern side is drier (200 mm per year) (del-Arco et al., 2006). La Palma, more oceanic and humid (rainfall exceeds 700 mm in most of the area), is the north-westernmost of the Canary Islands and has a wide variety of habitats (23 bioclimatics belts) (del-Arco et al., 1999). Lanzarote is the north-easternmost and closest island to Africa coast (100 km). The low altitude does not allow the development of trade wind clouds. Therefore, annual precipitation is below 200 mm and a limited number of bioclimatic belts (8) are defined (Reyes-Betancort et al., 2001).
During a study of yeast communities associated with grape berries in vineyards and wineries in Lanzarote, Tenerife and La Palma (Canary Islands, Spain), conventional taxonomic tests and routine PCR restriction fragment length polymorphism analysis of the rDNA 5.8S-internal transcribed spacer region (PCR-ITS-RFLP) were used for identification. A group of novel yeast strains with identical PCR-ITS-RFLP patterns was detected in Lanzarote. Analysis of D1/D2 domains of the large subunit (LSU) rDNA and the 5.8-ITS sequences of seven isolates indicated that these strains represented a distinct species closely related to Lachancea meyersii and Lachancea nothofagi. On the basis of phenotypic and phylogenetic analysis, a novel species of Lachancea, Lachancea lanzarotensis sp. nov., is proposed.

Methods

Sample collection and yeasts isolation. Collections were conducted in vineyards and wineries located in different bioclimatic conditions of the Canary Islands. Samples of healthy grapes and wineries were obtained from Lanzarote, Tenerife and La Palma. Grapes from 34 vineyards and samples from 23 wineries were collected during the 2009 harvest season, taking into account the bioclimatic factors of each island. In Tenerife, bunches were harvested from 18 vineyards belonging to three different zones and seven different bioclimatic belts ranging from 60 to 1175 m above sea level (m.a.s.l.). Vineyards sampled from this island are included in three different bioclimatic belts ranging from 188 to 390 m.a.s.l.

Ten grape bunches each from red (Listán Negro) and white grape varieties (Listán Blanco, in Tenerife and La Palma or Malvasía, in Lanzarote) were randomly and aseptically collected from each vineyard and stored in sterile plastic bags. All the samples were kept cool during transport to the laboratory and were then directly processed. Each grape bunch was homogenized in a Stomacher for 2 min and 100 µl of the resulting must was plated on Dichloran Rose Bengal Chloramphenicol agar (DRBC) plates and incubated at 25 °C for 48 h. A total of ten yeast colonies from each plate (corresponding to each bunch) were randomly isolated.

In addition a pool of musts elaborated with ten bunches from each variety and vineyard was made. From each pool, aseptic microvinifications were performed in 450 ml sterile bottles with 200 ml must. At the end of the fermentation process, 40 random yeast colonies obtained in DRBC were isolated from each microvinification.

Wineries samples were obtained at the beginning, middle and end of the fermentation process, from the same regions described above. From each sample 40 random yeast colonies were isolated from DRBC medium. Subcultivations were performed on YPD agar at 25 °C for 3 days and were subsequently preserved in 30 % (w/v) glycerol at −80 °C.

Yeast identification and characterization. Conventional taxonomic tests and routine PCR-ITS-RFLP (Esteve-Zarzoso et al., 1999) were used for identification. A group of novel yeast strains with identical PCR-ITS-RFLP patterns were detected in samples from Lanzarote and seven strains were selected for phenotypic characterization and sequence analysis (Table 1). Physiological, biochemical and ascospore examination of the yeasts were carried out as described by Yarrow (1998). For Gorodkowa and V8 agar media, used to induce the sporation, two different formulations of each were used: Gorodkowa A agar (0.1 % glucose, 0.5 % NaCl, 1 % peptone, 2 % agar), Gorodkowa B agar (0.25 % glucose, 0.5 % NaCl, 1 % malt extract, 2 % agar), Vegetable juice agar 1 (50 % V8 vegetable juice, 0.7 % yeast extract, 2 % agar) and V8 juice agar (Samson et al., 2010) (17.5 % V8 vegetable juice, 0.3 % CaCO₃, 0.001 % ZnSO₄·7H₂O, 0.0005 % CuSO₄·5H₂O, 2 % agar). All assimilation and fermentation tests and routine PCR restriction fragment length poly-

Table 1. Origin of strains of Lachancea lanzarotensis sp. nov. isolated from grape berries, microvinifications and wineries of Lanzarote, Canary Islands

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Sampling area</th>
<th>Bioclimatic characteristic</th>
<th>Altitude (m.a.s.l.)</th>
<th>Coordinates (Lat., Long.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5BA-1</td>
<td>FGM (Mv/LB)</td>
<td>La Geria</td>
<td>LSa-Xeric-UI</td>
<td>326</td>
<td>28.9764°, −13.6917°</td>
</tr>
<tr>
<td>L2C-15'</td>
<td>Vineyards (LN grapes)</td>
<td>Tinaj</td>
<td>UA-Desertic-UI</td>
<td>276</td>
<td>29.0440°, −13.6738°</td>
</tr>
<tr>
<td>CMV1-9</td>
<td>FGM (Mv)</td>
<td>Tinaj</td>
<td>UA-Desertic-UI</td>
<td>207</td>
<td>29.0620°, −13.6723°</td>
</tr>
<tr>
<td>12BA-11</td>
<td>FGM (LN)</td>
<td>Tinaj</td>
<td>UA-Desertic-UI</td>
<td>202</td>
<td>29.0656°, −13.6777°</td>
</tr>
<tr>
<td>FLN2-4</td>
<td>FGM (LN)</td>
<td>Mague</td>
<td>UA-Desertic-UI</td>
<td>196</td>
<td>29.1731°, −13.4717°</td>
</tr>
<tr>
<td>GMV1-7</td>
<td>FGM (Mv)</td>
<td>Ye</td>
<td>LSa-Xeric-UI</td>
<td>384</td>
<td>29.1937°, −13.4830°</td>
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<tr>
<td>GMV1-4</td>
<td>FGM (Mv)</td>
<td>Ye</td>
<td>LSa-Xeric-UI</td>
<td>392</td>
<td>29.1931°, −13.4818°</td>
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</table>
tests were performed at 25 °C in duplicate and results were recorded after 1, 2 and 3 weeks.

**DNA sequence analysis.** Nucleotide sequences of the D1/D2 domain of the LSU rDNA gene and 5.8S-ITS region of the strains L2C-15T, GMV1-7, GMV1-4, GMV1-7, FLN2-4, 5BA-1 and 12BA-11 were determined using the methods described by Kurtzman & Robnett (1998). Sequences were deposited in the GenBank database and the strains were deposited in the CBS and CECT culture collections as shown in Table 1. Comparisons with sequences from GenBank were performed using BLASTN (Altschul et al., 1997). The sequences were initially aligned using the multiple alignment program CLUSTAL W version 2.0 (Larkin et al., 2007). The phylogenetic tree was constructed using the neighbour-joining method (based on 1000 bootstrap iterations) in MEGA version 5 software (Tamura et al., 2011).

**Results and Discussion**

All strains tested exhibited similar physiological and morphological characteristics. However some differences were observed between strains in fermentation and assimilation tests for certain compounds. Strain GMV1-4 fermented D-galactose and α,α′-trehalose, but strains L2C-15T, GMV1-7, 12BA-11, 5BA-1, FLN2-4 and CMV1-9 were not fermentative. Strain L2C-15T did not assimilate xylitol, but the other strains did. Strain GMV1-7 was positive for assimilation of 2-keto-D-gluconate, but the other strains were negative. Strains GMV1-7, CMV1-9, GMV1-4, FLN2-4 were positive for growth in ethanol, but strains L2C-15T, 12BA-11 and 5BA-1 had a weak growth.

A comparison of chemotaxonomic properties of *L. lanzarotensis* sp. nov. with those of other recognized species of the genus *Lachancea* are shown in Table 2. *L. lanzarotensis* sp. nov., *L. meyersii* and *L. nothofagi*, displayed similar morphological and physiological characteristics. However, *L. lanzarotensis* sp. nov. could be distinguished from *L. meyersii* by its ability to assimilate D-galactose and from *L. nothofagi* by the ability to assimilate ethanol as a sole carbon source. For accurate identification of these species, sequencing the 5.8S-ITS rDNA regions is needed. As an alternative to sequencing, a restriction analysis PCR-ITS-RFLP can be performed. Restriction of the 5.8S-ITS rDNA regions with enzymes HinfI or Ddel separates *L. lanzarotensis* sp. nov. from *L. meyersii* and *L. nothofagi*. The three species have the same amplified product size (680 bp) with primers ITS1 and ITS4. With the enzyme HinfI the PCR-ITS-RFLP pattern of *L. lanzarotensis* sp. nov. has five fragments (332 + 8 + 150 + 75 + 115 bp), while *L. meyersii* and *L. nothofagi* have four fragments (332 + 8 + 150 + 190 bp). With the enzyme Ddel *L. lanzarotensis* sp. nov. produces two fragments (486 + 194 bp), while *L. meyersii* and *L. nothofagi* produce three fragments (415 + 71 + 194 bp). The PCR-ITS-RFLP technique is reproducible, rapid and easy to use for yeast species identification. This study also demonstrates its potential use in the detection of possible new species during yeast population diversity analysis.

The seven strains were sequenced in the D1/D2 domain of the LSU rDNA gene. Strains L2C-15T, GMV1-4, GMV1-7 and CMV1-9 were found to share identical sequences, however one nucleotide insertion was found in strains 5BA-1 and FLN2-4, and two nucleotide insertions in strain 12BA-11. A similar analysis was performed by 5.8S-ITS rDNA sequencing and in this case all the isolates had identical sequences.

The phylogenetic tree constructed using the neighbour-joining method based on D1/D2 sequences showed that the novel species clustered with *L. meyersii* NRRL Y-27269T (GenBank accession no. AY645656) and *L. nothofagi* NRRL Y-48670T (GQ411403) (Fig. 1). Strain L2C-15T showed 98.3 % sequence similarity (8 substitutions + 2 gaps) with *L. meyersii* and 97.3 % sequence similarity (16 substitutions) with *L. nothofagi*. A similar analysis was performed with the 5.8S-ITS rDNA sequences and the results demonstrated that 5.8S-ITS tree was congruent with D1/D2 tree. In the case of ITS locus, strain L2C-15T showed 22 (96.24 % sequence similarity with 2 gaps) and 29 (95.05 % sequence similarity with 3 gaps) substitutions with *L. meyersii* and *L. nothofagi*, respectively.

**Table 2. Comparison of phenotypic properties of *Lachancea lanzarotensis* sp. nov. and other recognized species of the genus *Lachancea***

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>6</th>
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<tr>
<td>D-Galactose</td>
<td>+/-</td>
<td>-</td>
<td>D</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>L-Sorbose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>D</td>
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<tr>
<td>Maltoose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>W</td>
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<tr>
<td>Melibiose</td>
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<td>Inulin</td>
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<td>Glycerol</td>
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<td>+</td>
<td>D</td>
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<td>V</td>
<td>V</td>
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<tr>
<td>DL-Lactate</td>
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<td>+</td>
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<td>Succinate</td>
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<td>V</td>
<td>-</td>
<td>V</td>
<td>-</td>
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<tr>
<td>Ethanol</td>
<td>+/-</td>
<td>+/D</td>
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<td>-</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>V</td>
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<tr>
<td>0.01 % Cycloheximide</td>
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<td>+</td>
<td>+</td>
<td>V</td>
<td>V</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Species: 1, *Lachancea lanzarotensis* sp. nov. (data from this study); 2, *Lachancea meyersii* (data from Fell et al., 2004); 3, *Lachancea nothofagi* (Mestre et al., 2010); 4, *Lachancea daisiensis* (Lee et al., 2009); 5, *Lachancea thermotolerans* (Lachance, 2011); 6, *Lachancea waltii* (Lachance, 2011); 7, *Lachancea kuyveri* (Lachance, 2011); 8, *Lachancea cidri* (Lachance, 2011); 9, *Lachancea fermentati* (Lachance, 2011); 10, *Lachancea mirandina* (Pereira et al., 2011). +, Positive; −, negative; W, weak; V, variable; D, delayed growth; s, slow growth.
plants in Taiwan; a difference in one position (one insertion) with a group of strains isolated from grapes in Portugal and identified as *Lachancea thermotolerans* (GenBank accession no. EU541357) by their phenotypic characteristics (Barata et al., 2008); and three positions difference (three substitutions) with *Lachancea* sp. CBS 6924 (GenBank accession no. EF463105) isolated by van der Walt from garden soil in South Africa (Fig. 1). According to Kurtzman & Robnett (1998), yeast strains with 0–3 nt difference are conspecific or sister species, for this reason and using the proposed phenetic standard these isolates could be considered conspecific.

*Lachance* (2011) discusses in detail the phylogenetic position of strain CBS 6924 in the genus *Lachancea*, and concludes that this strain deposited in 1975 by van der Walt as a specimen of *Kluyveromyces thermotolerans* is closely related to *L. meyersii*, but it is unclear whether it represents a distinct species. According to our phylogenetic tree constructed with the sequences available in GenBank (D1/D2 and 5.8S-ITS) this strain could be considered conspecific with the proposed species. However this investigation did not attempt to revise previous taxonomy.

Isolation of the strains of *L. lanzarotensis* sp. nov. only from Lanzarote could probably indicate that the proposed species has better capacity for adaptation or tolerance to desertic bioclimatic conditions. However, in Tenerife and La Palma samples were obtained from xeric bioclimatic areas on the southern sides of these islands and the proposed species was not detected. For this reason, it is probable that the population of this species is lower in Tenerife and La Palma, and the sampling protocol employed (10 randomly isolated colonies per bunch) did not allow detection. Also, it must be remembered that this species was isolated by other authors from grapes, plants and soil in different environments (Portugal, Taiwan and South Africa), which shows the potential distribution and adaptability in distinct climatic regions.

Based on phenotypic and phylogenetic analysis, strains L2C-15\(^{\dagger}\), GMV1-4, GMV1-7, CMV1-9, 5BA-1, FLN2-4 and 12BA-11 represent a novel species of the genus *Lachancea*, for which the name *Lachancea lanzarotensis* sp. nov., is proposed.

**Latin diagnosis of Lachancea lanzarotensis**

González, Alcoba-Flórez & Laich sp. nov.

succinicum, acidum citricum, methanolum, acidum levulinici-
cum, acidum malicum et N-acetyl-d-glucosaminum. Non
assimilantur nitratum, nitritum, L-lysimum, glucosaminum et
D-prolimum. Amyllum non formatur. Ureum non hydrolysat.
Vitaminum externum ad crescentiam necessarium est. Non
crescit in medio cum 0.1 % cycloheximido addito. Non crescit
in substrato 16 % sal/5 % glucosum continent. Non crescit in
60 % glucosum addito. Maxima temperature crescentiae: 32
°C.

_Typus stirpis_ L2C-15^T (=CBS 12615^T = CECT 13066^T)
isolatus ex uva (Vitis vinifera L. cv. Listán Negro) in insula
Lanzarote (Hispania). Colección Española de Cultivos Tipo
(CECT), Valencia, Spain, et Centraalbureau voor Schimmelcultures
(CBS), Utrecht, The Netherlands, _deposita est._

**Description of _Lachancea lanzarotensis_**

_González, Alcoba-Flórez & Laich sp. nov._

_Lachancea lanzarotensis_ (lan.za.ro.ten’sis. N.L. fem. adj.
_lanzarotensis_ of or belonging to Lanzarote, Spain).

After 7 days on 5 % Malt extract (ME5 %) and YM agar at
25 °C, colonies (1.7–2.6 mm in diameter) are creamy,
butyrous, convex, centrally heaped up (especially on YM)
with circular and entire margins, and smooth and glistening
surfaces (on ME5 % some isolates are matt) (Fig. 2a). After
growth in ME5 % and YM broth at 25 °C for 2 days, cells are
usually spherical to subspheroidal (2.9–5.8 μm in diameter)
and occur singly or in pairs (Fig. 2b). Budding is
multilateral. After 1 month at 25 °C, sediment formation
is observed. Pseudohyphae and hyphae are not observed on
any of the culture media tested. Abundant ascosporulation
was observed after 8 days at 25 °C on Gorodkowa B agar, V8
juice agar, ME5 % agar and Acetate agar 1. A minor amount
of ascospores were observed on Glucose-Peptone-Yeast
extract agar, Restricted growth agar, Gorodkowa A agar and
Yeast extract-2 % Glucose agar. Ascospores were not
observed on Vegetable juice agar 1, Acetate agar 2 and
YM-2 % sodium chloride agar. The asci are formed by
conjugation between independent cells or autogamously by
bud-mother cell conjugation. Ascospores (1–2 per ascus) are
spherical (2.3–2.8 μm in diameter) with smooth walls and
are quickly liberated from the ascus (Fig. 2c & d). Ferments
glucose, galactose (variable), malate, sucrose and α,α-
trehalose (variable), but not lactose, inulin or starch.
Assimilates glucose, galactose (weak), sucrose, maltose,
α,α-trehalose, methyl α-D-glucoside, raffinose, melezitose,
glycerol (variable), xylitol (variable), D-glucitol (weak), D-
mannitol, 2-keto-D-gluconate (variable), D-gluconate
(weak), ethanol and palatinose, but not L-sorbose, D-
glucosamine, D-ribose, D-xylone, L-arabinose, D-arabinose,
L-rhamnose, cellobiose, salicin, arbutin, melibiose, lactose,
inulin, starch, erythritol, ribitol, galactitol, myo-Inositol, 5-
keto-D-gluconate, D-gluconate, D-galacturonate, DL-lact-
ate, succinate, citrate, levulinate, L-malic acid and N-
acetylglucosamine. Nitrate, nitrite, L-lysine, glucosamine
and D-proline are not assimilated. Does not grow in
vitamin-free media. Grows in 50 % glucose, but not in
60 % glucose or in 16 % NaCl. Growth on 10 % NaCl plus
5 % glucose is positive in most strains. All strains are
sensitive to 0.01 % cycloheximide and do not grow in
medium containing 1 % (v/v) acetic acid or methanol.
The maximum temperature for growth is 32 °C. Urease
hydrolysis and diazonium blue B reactions are negative. No
starch-like substance is produced. Acid production is weak.

**Fig. 2.** _Lachancea lanzarotensis_ sp. nov. strain L2C-15^T. (a) Colonies on ME5 % agar after 7 days at 25 °C. (b) Budding cells
in ME5 % broth for 2 days at 25 °C. (c–d) Ascospores produced on V8 juice (c) and ME5 % agar (d) after 8 days at 25 °C.
Microscopic characteristics were examined under differential interference contrast (Nomarski) optical microscope. Bars, 2 mm
(a) and 10 μm (b–d).
The type strain, L2C-15\(^T\) (=CBS 12615\(^T\)=CECT 13066\(^T\)) was isolated from grape berries of *Vitis vinifera* L. cv. Listán Negro red grape variety in Tinajo, Lanzarote, Canary Islands, Spain, in 2009. Additional reference strains of *L. lanzarotensis* are 5BA-1 (=CBS 12616=CECT 13067), CMV1-9 (=CBS 12621=CECT 13072), 12BA-11 (=CBS 12620=CECT 13068), FLN2-4 (=CBS 12619=CECT 13069), GMV1-7 (=CBS 12617=CECT 13071) and GMV1-4 (=CBS 12618=CECT 13070). More details are given in Table 1.

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