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UNCOVERING TAXONOMIC CONFUSION IN *COSTUS*: MOLECULAR EVIDENCE FOR MISIDENTIFICATION BETWEEN *C. afer* AND *C. lucanusianus* IN THE BOGOR BOTANIC GARDEN LIVING COLLECTION

Rifqi Nur Mahmudah[1], Baiq Farhatul Wahidah[1*]

[1]Program Study of Biology, Faculty of Science and Technology,
Universitas Islam Negeri Walisongo, Semarang, 50185, Indonesia
Correspondence: baiqfarhatulwahidah@walisongo.ac.id

ABSTRACT

Rifqi Nur Mahmudah & Baiq Farhatul Wahidah 2025. Mengungkap Kekeliruan Taksonomi pada *Costus*: Bukti Molekuler Atas Kesalahan Identifikasi antara *C. afer* dan *C. lucanusianus* dalam Koleksi Hidup Kebun Raya Bogor. Floribunda 8(1): 32 – 38 — Identifikasi spesies yang akurat sangat penting untuk menjaga integritas koleksi botani, terutama ketika kemiripan morfologi menyebabkan kebingungan taksonomi. Penelitian ini mengkaji kesalahan identifikasi antara *Costus afer* dan *C. lucanusianus* pada koleksi hidup Kebun Raya Bogor dengan pendekatan molekuler. Sampel daun dari kedua takson diekstraksi DNAnya, diamplifikasi pada daerah ITS, dan disekuensing menggunakan metode Sanger. Hasil analisis BLAST menunjukkan bahwa seluruh sekuen berasal dari genus *Costus* dengan nilai identitas tinggi dan E-value sebesar 0.0, menandakan homologi yang signifikan. Penyejajaran sekuen sepanjang 731 bp menunjukkan 208 situs variabel dan 500 situs konservatif. Analisis filogenetik menggunakan metode Maximum Likelihood (MEGA 12) menunjukkan bahwa kedua spesimen dari Kebun Raya Bogor mengelompok dalam klad *C. afer*. Menariknya, sampel yang dilabeli sebagai *C. lucanusianus* justru berkerabat dekat dengan *C. afer* (AY972934.1 and KJ011425.1), mengindikasikan adanya kesalahan identifikasi. Hasil ini sejalan dengan kajian morfologi sebelumnya dan menegaskan efektivitas barcoding DNA dalam menyelesaikan ambiguitas taksonomi. Studi ini menekankan pentingnya verifikasi molekuler dalam pengelolaan koleksi kebun botani untuk mendukung dokumentasi spesies yang akurat dan konservasi yang berbasis data.

Kata kunci: Analisis BLAST, DNA barcoding, filogenetik, kurasi kebun raya, sekuen ITS.

Rifqi Nur Mahmudah & Baiq Farhatul Wahidah 2025. Uncovering Taxonomic Confusion in *Costus*: Molecular Evidence for Misidentification Between *C. afer* and *C. lucanusianus* in The Bogor Botanic Garden Living Collection. Floribunda 8(1): 32 – 38 — Accurate species identification is critical for the integrity of botanical collections, especially when morphological similarities lead to taxonomic confusion. This study investigates the misidentification between *Costus afer* and *C. lucanusianus* in the living collection of the Bogor Botanic Garden using molecular evidence. Leaf samples of both taxa were subjected to DNA extraction, PCR amplification of the ITS region, and Sanger sequencing. BLAST analysis confirmed all sequences belonged to the genus *Costus*, with high identity scores and E-values of 0.0, indicating significant homology. Sequence alignment revealed 208 variable and 500 conserved sites across 731 bp. Phylogenetic analysis using the Maximum Likelihood method (MEGA 12) showed that both Bogor accessions clustered within the *C. afer* clade. Notably, the sample labeled *C. lucanusianus* grouped with *C. afer* (AY972934.1 and

KJ011425.1), suggesting misidentification. These results are consistent with previous morphological assessments and highlight the effectiveness of DNA barcoding in resolving taxonomic ambiguities. The study underscores the importance of molecular verification in botanical gardens to support accurate species documentation and conservation.

Keywords: BLAST analysis, botanic garden curation, DNA barcoding, ITS region, phylogenetic.

The genus *Costus* L. (Costaceae) represents a taxonomically challenging group of spiral gingers widely distributed across the tropical regions of Africa and the Americas, with its center of species diversity located in the Neotropics (Specht & Stevenson, 2006; André *et al*., 2016). In Java, recent taxonomic studies have recorded nine species of *Costus* (Irsyam *et al*., 2024). Among these, *C. afer* Ker Gawl., a species native to tropical Africa, has drawn particular attention due to its status as a naturalized plant in several areas of West Java, including Maribaya, Dago Atas, and the IPB Dramaga Campus (Irsyam *et al*., 2019). The naturalization of *C. afer* in Java is notable, as it suggests successful ecological adaptation outside its native range, likely facilitated by favorable tropical climatic conditions and potential anthropogenic introduction through ornamental or medicinal plant trade. Its establishment in disturbed habitats such as roadsides, gardens, and forest edges indicates a degree of ecological plasticity, which may enable the species to persist and spread in non-native environments. This phenomenon underscores the importance of monitoring non-native species introductions, as naturalized taxa can have ecological implications, including competition with native flora, alteration of local plant communities, and challenges in taxonomic identification, especially within living collections.

A primary concern raised by Irsyam *et al*. (2019) is the taxonomic ambiguity between *C. afer* and *C. lucanusianus* J. Braun & K. Schum., due to overlapping vegetative and floral characters. The two species share morphological features such as paired flowers per bract, inflorescence shape, and general leaf architecture, which have led to frequent misidentifications. Notably, earlier herbarium records from Bogor Botanic Garden, such as the specimen Martati 61, were misidentified as *C. lucanusianus* despite exhibiting morphological characters that align more closely with *C. afer* (Irsyam *et al*., 2019). Recent taxonomic revisions have clarified the diagnostic traits between the two, including differences in floral lip coloration, indumentum of the ligule base, and bract morphology (Maas-van de Kamer *et al*., 2016), yet field and herbarium studies continue to reveal inconsistency in identification.

Given the limitations of morphological data in resolving taxonomic ambiguities—especially within living collections where intraspecific variation may be influenced by environmental factors—a molecular approach is essential. DNA-based identification offers an independent and robust framework for species delimitation, enabling the re-evaluation of potentially misidentified accessions and enhancing taxonomic accuracy. Among the commonly used molecular markers in plant species identification are nuclear and chloroplast regions such as the internal transcribed spacer (ITS), *mat*K, *rbc*L, *trn*L–F, and *psb*A–*trn*H (Devi *et al*., 2022). These markers vary in their levels of resolution, conservation, and amplification efficiency. In this study, the ITS region was selected due to its high mutation rate, strong discriminatory power at the species level, and ease of amplification across a wide range of taxa. As a nuclear marker, ITS complements chloroplast markers and is particularly useful in detecting cryptic diversity or hybridization events. Additionally, the abundance of ITS reference sequences in public databases facilitates accurate comparative analysis, making it a valuable tool for clarifying taxonomic uncertainty in morphologically variable plant groups.

This study aims to apply molecular markers to verify the identity of *Costus* specimens

cultivated in the Bogor Botanic Garden, with particular focus on distinguishing *C. afer* from *C. lucanusianus*, thereby contributing to a more accurate understanding of species composition and supporting the integrity of ex situ conservation efforts in botanical gardens (Irsyam *et al*., 2019).

MATERIALS AND METHODS

Plant Material

Leaf samples of *C. afer* XI.B.V.125 and *C. lucanusianus* XI.B.II.33 were collected from the living collection of the Bogor Botanic Garden, Indonesia (Fig. 1). Young, healthy leaf tissues were sampled in the field and immediately placed in silica gel for rapid desiccation, following the protocol described by Harenčár *et al*. (2023) to preserve DNA integrity for downstream molecular analysis.

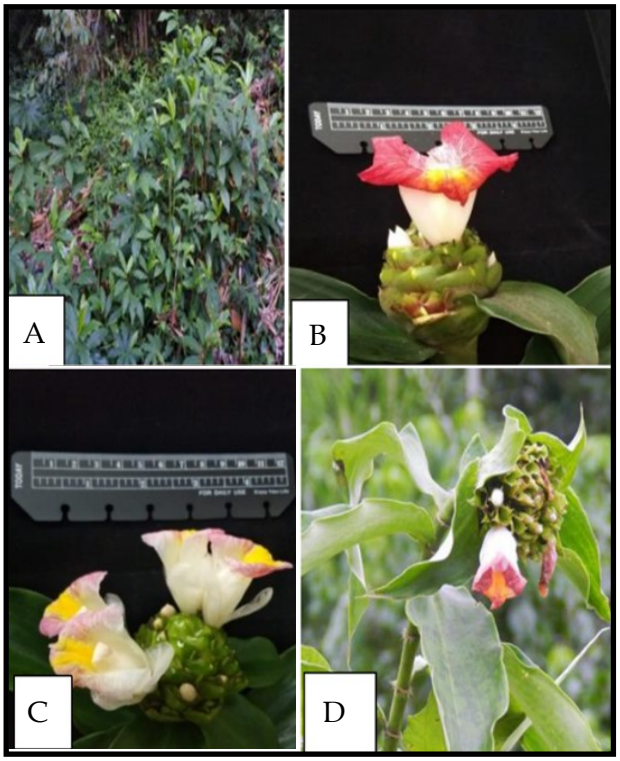

Figure 1. Materials of *Costus afer* (A-B) and *Costus lucanusianus* (C-D) used in this study. A. Habit, B. Inflorescense showing one flower bloom, C. Habit, and D. Inflorescense showing four flowers bloom.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from fresh leaf tissues using the Tiangen Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. PCR was performed in a 50 µL reaction volume containing 5 µL of DNA template, 1.5 µL of each ITS forward and reverse primer (Sun *et al*., 1994), 17 µL ddH_2O, and 25 µL MyTaq DNA polymerase mix. The thermal cycling conditions were: initial denaturation at 95°C for 3 minutes; 35 cycles of denaturation at 95° C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 45 seconds; followed by a final extension at 72°C for 5 minutes.

Agarose gel (1%) was prepared by dissolving 0.8 g agarose in 80 mL of 1× TAE buffer, heating the solution, and adding 1 µL of GelRed. PCR products and a 1 kb DNA ladder were loaded onto the gel and electrophoresed at 100 V for 30 minutes. DNA bands were visualized using a Gel Doc EZ Imager (Bio-Rad). Amplified DNA products were purified and submitted to 1st Base Laboratories (Singapore) via PT Genetika Science Indonesia for bidirectional Sanger sequencing using ITS primers.

Data Analysis

Forward and reverse sequence chromatograms were trimmed and assembled into contigs using MEGA 12 to obtain high-quality consensus sequences (Kumar *et al*., 2024). Species identification was confirmed by BLAST analysis against the NCBI GenBank database. Multiple sequence alignments, including the generated sequences and BLAST hits, were performed in MEGA 12 and further analysis was performed using IQ-TREE (Nguyen *et al*., 2015). A phylogenetic tree incorporating a total of 31 sequences, including *Tapeinochilos queenslandiae* (F.M.Bailey) K.Schum. (KJ011480.1) as outgroup, was reconstructed using the Maximum Likelihood method with the TPM3u+F+G4 model based on ModelFinder (Kalyaanamoorthy *et al*., 2017). The

node support was evaluated through ultra fastbootstrap analysis with 1,000 replicates (Hoang *et al*., 2018). Phylogenetic tree construction was performed using Interactive Tree of Life (iTOL, Letunic & Bork 2021).

RESULTS AND DISCUSSION

The ITS region of both *C. afer* and *C. lucanusianus* was successfully amplified using ITS primers, as shown in Figure 2. The PCR product of *C. afer* exhibited a thick and intense band, indicative of high DNA concentration. This observation is consistent with Indriyani (2017), who noted that band intensity in gel electrophoresis is directly proportional to DNA concentration. Meanwhile, the *C. lucanusianus* sample produced a single, distinct, and well-defined band, confirming efficient and specific amplification.

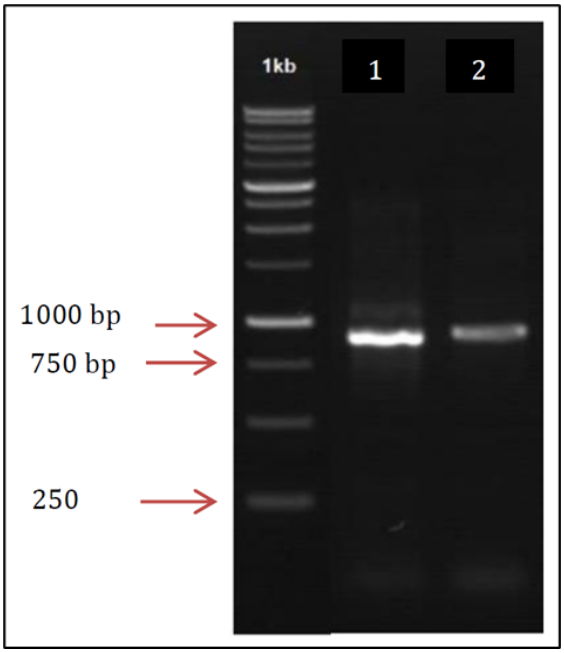

Figure 2. PCR product visualization. 1=*Costus afer*, 2=*Costus lucanusianus* with 1 kb ladder as standard.

BLAST analysis of the sequenced ITS regions confirmed that all resulting sequences matched members of the genus *Costus*. The five top-ranking hits for each sample shown in Tables 1 were selected based on the highest percentage identity (Perc. Ident), lowest E-value, and maximum query coverage. The E-value scores of 0.0 across all hits indicate highly significant alignments, as values below 0.05 are considered statistically robust (Frederick *et al*., 2003). High percentage identity suggests strong sequence homology, while full query coverage strengthens the confidence in species-level matches.

Table 1. The top 5 NCBI BLAST result for *Costus afer* and *Costus lucanusianus* from BBG living collection

Species Name	NCBI Accession Number	% Identity (%)	E value	Query Cover (%)
Costus afer XI.B.V.125				
Costus pulverulentus	AY673070.1	97.73	0	89
Costus afer	KJ011425.1	98.61	0	85
Costus claviger	AY994740.1	98.18	0	85
Costus lucanusianus	KJ011455.1	98.18	0	84
Costus vinosus	KJ011472.1	98.44	0	84
Costus lucanusianus XI.B.II.33				
Costus pulverulentus	AY673070.1	98.00	0	89
Costus afer	KJ011425.1	99.17	0	85
Costus lucanusianus	KJ011455.1	98.46	0	84
Costus claviger	AY994740.1	98.18	0	85
Costus vinosus	KJ011472.1	98.44	0	84

The aligned ITS sequences, totaling 731 base pairs, revealed the presence of insertions/deletions (indels) or gaps. These gaps are commonly associated with genetic variations and evolutionary events such as indels or structural rearrangements (Dharmayanti, 2011). The alignment also showed 208 variable sites and 500 conserved sites, highlighting the informative nature of the ITS region, especially given its location within the nuclear genome (Su'udi, 2018).

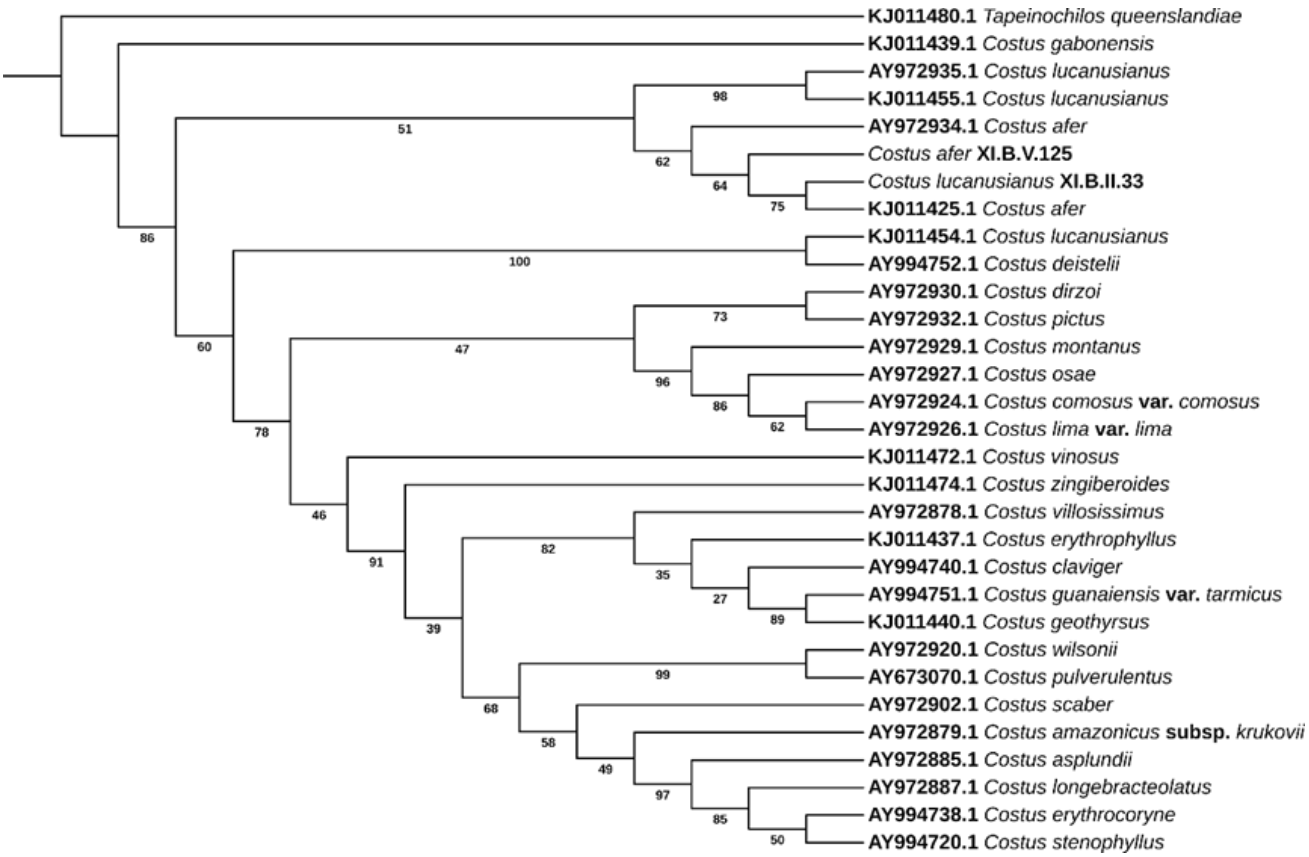

Figure 3. Phylogenetic tree constructed using Maximum Likelihood method with xxx parameter model and 1000× bootstraps.

Surprisingly, the accession labeled as *C. lucanusianus* (XI.B.II.33), also from the Bogor Botanic Garden, does not group with any other *C. lucanusianus* sequences. Instead, it is nested within the *C. afer* clade, strongly suggesting that the sample has been misidentified and should be reclassified as *C. afer*. This molecular evidence is consistent with previous morphological observations that documented overlapping floral and foliar traits between the two taxa (Irsyam *et al.*, 2019). The clustering pattern reinforces the necessity of integrating molecular data in curating living plant collections, especially for taxa with high morphological similarity.

An intriguing observation emerges from the position of *C. lucanusianus* KJ011454.1, which does not cluster with the other *C. lucanusianus* accessions. Instead, it groups closely with *C. deistelii* AY994744.1, a recognized synonym of *C. afer*. This unexpected relationship may reflect potential cryptic speciation within the *C. afer/C. lucanusianus* complex or mislabeling in the sequence database. Given that *C. deistelii* has historically been merged under *C. afer* based on morphological grounds (POWO 2025), its close phylogenetic affinity with *C. lucanusianus* KJ011454.1 raises new questions regarding the genomic boundaries of these taxa. This pattern warrants further

investigation involving broader geographic sampling, nuclear and plastid markers, and potentially population-level studies to detect lineage sorting or introgression.

Moreover, the presence of two *C. lucanusianus* accessions (AY972935.1 and KJ011455.1) forming a highly supported monophyletic clade suggests a stable genetic identity for at least one lineage of *C. lucanusianus*. The contrast between this clade and the divergent position of *C. lucanusianus* KJ011454.1 points to possible intraspecific genetic divergence or historical taxonomic confusion.

According to Maas (1979), *C. lucanusianus* exhibits a white labellum with prominent dark purple stripes. This floral trait was later revisited by Maas-van de Kamer *et al*. (2016), who emphasized its diagnostic value in distinguishing *C. lucanusianus* from *C. afer*. In contrast, *C. afer* typically has a plain white labellum or one suffused with varying shades of pale to deep pink. The margin of the labellum in *C. lucanusianus* is characteristically deep red, setting it apart from *C. afer*. Aside from flower color, both species differ in several other morphological features, such as the presence of basal hairs on the leaf ligule, leaf blade and base shape, the color and type of indumentum on the abaxial leaf surface, inflorescence form, and the orientation of calyx lobes (Irsyam *et al*., 2019).

The findings highlight the need for taxonomic re-evaluation of herbarium and living collections, particularly within morphologically complex genera such as *Costus*. Molecular data not only clarify misidentifications but also uncover hidden evolutionary signals that might otherwise be overlooked. For Bogor Botanic Garden and similar institutions, integrating DNA-based verification into their curation workflows is essential to ensure accuracy, facilitate biodiversity assessments, and support future taxonomic revisions.

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