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# Design of genus-specific semi-nested primers for simple and accurate identification of *Enterobacter* strains

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## Abstract

**Background** The genus *Enterobacter*, in the family *Enterobacteriaceae*, is of both clinical and environmental importance. This genus has undergone frequent taxonomic changes, making it challenging to identify taxa even at genus level. This study aimed to design *Enterobacter* genus-specific primers that can be used for simple PCR identification of large sets of putative *Enterobacter* isolates.

**Results** Comparative genomic approaches were employed to identify genes that were universally present on *Enterobacter* genomes but absent from the genomes of other members of the family *Enterobacteriaceae*, based on an initial set of 89 genomes. The presence of these genes was further confirmed in 4,276 *Enterobacter* RefSeq genomes. While no strictly genus-specific genes were identified, the *hpaB* gene demonstrated a restricted distribution outside of the genus *Enterobacter*. Semi-nested primers were designed for *hpaB* and its flanking gene *hpaC* (*hpaBC*) and evaluated on 123 strains in single-tube PCR reactions. All taxa showing positive reactions belonged to the genus *Enterobacter*. For *Enterobacter* strains the PCR yielded two amplicons at 110 bp and at 370 bp, while strains only displaying the 110 bp amplicon were classified as *Leclercia pneumoniae*. A blind-test on 120 strains accessioned as *Enterobacter* sp. from the USDA-ARS culture collection (NRRL), revealed that one third of the strains had an incorrect genus assignment. Comparison of gene trees of the *hpaBC* fragment sequences with marker genes frequently used for single-gene barcoding or multi-locus sequence analysis (MLSA) further demonstrated its potential for preliminary species identification.

**Conclusions** The nested PCR assay represents a rapid and cost-effective approach for preliminary identification of *Enterobacter* species. As the primer design was based on large-scale genomic comparison, including currently undescribed species clades, it will remain valid even after taxonomic changes within the genus.

**Keywords** *Enterobacteriaceae*, *Leclercia pneumoniae*, PCR, Diagnostics

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## Background

The genus *Enterobacter* encompasses Gram-negative, rod-shaped, facultatively anaerobic bacteria that belong to the family *Enterobacteriaceae* [1]. Members of this genus are ubiquitous in nature and are frequently isolated from various sources including soil, water, sewage, plants, and from the intestines of animals and humans. In the latter hosts, it is known as an opportunistic, yet important, pathogen [1]. As such *Enterobacter* spp. are included in the "ESKAPE" group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), which were described as the most important antibiotic resistant bacterial pathogens causing nosocomial infections [2–4].

The genus *Enterobacter* was first proposed by E. Hormaeche and P. R. Edwards in 1960 based on biochemical characteristics [5]. With the advent of molecular tools, the taxonomy of this genus has been subjected to frequent changes. During the last decades several species were removed from this genus and assigned to other genera or were described as heterotypic synonyms [4–7]. One multi-locus sequence analysis (MLSA) study reclassified twelve species to the genera *Lelliottia*, *Pluralibacter*, *Kosakonia* and *Cronobacter* [8]. Similarly, new *Enterobacter* species continue to be described [9–12]. According to the list of prokaryotic names with standing in nomenclature (LPSN), the genus currently consists of 27 validly described species, 19 of which have been published in the last decade [13, 14]. Additionally, *E. adelaidei* was recently described as novel species but has not yet been validated by the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) [15]. The closest neighbors of the genus *Enterobacter* are *Leclercia* and *Lelliottia*, which together form the "*Enterobacter* clade" [16]. Recently, two new genera, *Huaxiibacter* and *Silvania*, were described as belonging to this clade [17, 18].

The frequent taxonomic changes within and around this genus and the close relationship of novel genera makes it challenging to reliably identify *Enterobacter* strains. Sequencing of the 16S rRNA gene is known to not be of limited use as sequences of different genera can not be separated sufficiently [19], while the databases have many misidentified organisms included, partly also due to taxonomic revisions [20]. The common use of biochemical panels in diagnostics, like BD Phoenix™ or VITEK-2® (bio-Merieux) are also of limited used as it can lead to false *Enterobacter* genus assignments [21, 22].

As an alternative to sequencing approaches, matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) is a powerful tool for identifying bacterial species, which is particularly used in routine diagnostics [23]. However, this method also has its limitations and can lead to misidentifications, especially when using pattern-matching approaches with manufacturer-provided databases, as these are usually overrepresented by clinical isolates and do not cover all species or even genera. For example, the current version of the SARAMIS® database only covers seven *Enterobacter* species and does not include the closely related genera *Huaxiibacter* and *Silvania*. Biomarker-based databases based on molecular masses of ribosomal proteins calculated from genome sequences have a high potential for identification [24, 25]. The most optimal approach currently is the use of whole-genome sequencing (WGS). Both approaches, MALDI-TOF MS and WGS require expensive devices and extended know-how and are therefore not an option for many labs,

As such, this study aimed to develop a simple PCR assay for the detection and assignment of isolates that are members of the genus *Enterobacter*. While specific PCR primers are available for some *Enterobacter* species [26, 27], none exist that target the entire genus, especially not after the recent large taxonomic changes. By using comprehensive comparative genomics approaches for target gene selection, we have developed a robust and cost-effective tool for *Enterobacter* identification.

## Results and discussion

### Comparative genomic analyses

To investigate the genus border of *Enterobacter*, a core gene phylogeny of 89 *Enterobacteriaceae* was generated (Fig. 1) using the pipeline UBCG2 (Up-to-date Bacterial Core Genes 2; [28]). The tree topology showed a clear separation of the "*Enterobacter* clade", encompassing the genera *Enterobacter*, *Huaxiibacter*, *Leclercia*, *Lelliottia*, *Silvania* and *"Pseudenterobacter"*, from other taxa in the *Enterobacteriaceae*. The protein sequences of all 89 *Enterobactericeae* taxa were compared using OrthoFinder [29]. The pan-genome encompassed a total of 12,244 orthogroups, with 732 of these being core to all 89 taxa, while 2,112 proteins were core to all taxa in the "*Enterobacter*" clade. The core proteome of the 29 taxa in the genus *Enterobacter* (Fig. 1) comprised 2,368 proteins. None of these core proteins were, however, exclusive to *Enterobacter* spp., with between 13 and 645 orthologues in the other 60 taxa. As such, the 35 orthogroups with orthologues in <25 of the non-*Enterobacter* taxa were analyzed further. Priority was then given to those proteins that were absent from the genera closest to *Enterobacter* in the core gene tree. No proteins were identified that were absent in all other members of the "*Enterobacter* clade", however, one protein, HpaB (a 4-hydroxyphenylacetate 3-monooxygenase oxidase component) was absent from the genomes of the closely related *Huaxiibacter chinensis* 155,047$^T$, *Lelliottia amnigena* LMG 2784$^T$, *Lelliottia nimipressuralis* CCUG 25,894$^T$

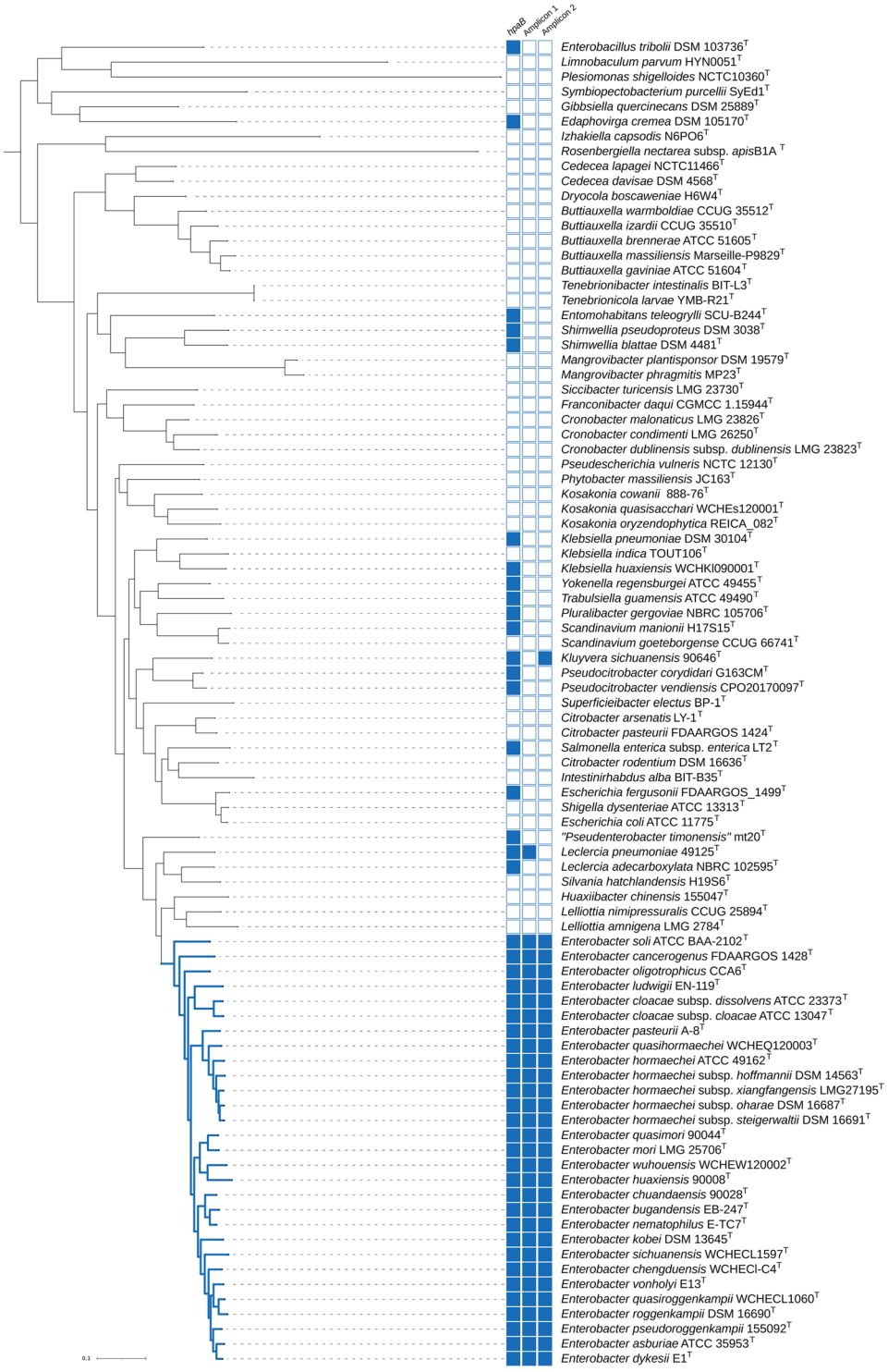


**Fig. 1** Core genome tree based on 81 bacterial core genes in the type strains of 29 *Enterobacter* species/subspecies (blue) and 60 other *Enterobacteriaceae* used for the comparative genomics. Filled squares show (1) Presence of the target gene *hpaB*, (2) Expected small PCR amplicon 1 in members of the genus, (3) Expected long PCR amplicon 2 in members of the genus

and *Silvania hatchlandensis* H19S6$^T$ (Fig. 1). As such, sequences of *hpaB*, along with its neighboring genes *hpaA* and *hpaC* were selected for primer design.

To confirm the presence of *hpaABC* on the genomes of a broader set of *Enterobacter* strains, the publicly available genomes of all *Enterobacter* strains (4,276 RefSeq assemblies) were downloaded from the NCBI database and subjected to local BLASTN analysis with the *hpaABC* sequence of *E. cloacae* ATCC 13,047$^T$ (CP001918.1 region 743,259 to 746,860) as query sequence. The region was absent from the genomes of only two *Enterobacter* strains, namely, *Enterobacter roggenkampii* FDAARGOS_523 (GCF_003812145.1) and *Enterobacter cloacae* Tembi-46 (GCA_024583295.1). As only 0.047% of the genomes are lacking the gene, it is assumed that it is part of the core genome of the genus and the lack thereof is due to genome incompleteness.

The BLASTN search with the *hpaABC* sequence of *E. cloacae* ATCC 13,047$^T$ against all non-Enterobacter genomes yielded in total 8,246 genomes, in which this fragment was present (Supplementary Table S1). This yielded 34 hits within the genus *Leclercia*, but none within the genera *Huaxiibacter*, *Silvania*, and *Lelliottia*. Other genera having the *hpaABC* region included other members of the *Enterobacteriaceae*, like *Klebsiella*, *Escherichia* or *Salmonella*.

#### Primer design and in silico testing

By removing redundant genomes from the set of 4,276 *Enterobacter* genomes, a subset of 1069 *Enterobacter* genomes was obtained, from which the *hpaABC* region was used for primer design. As no primer pair could be identified that excluded all other genera, a second forward primer was designed that should exclude *Leclercia pneumoniae*. The regions of the final primers hpa_287F (5'-GGCGCGCCATGATCTCY-3'), hpa_521F (5'-GCTGCRYTATTTCAGCAGCT-3') and hpa_625R (5'-ATGGTCGACCGCTGYATGTC-3') showed a high conservation among the *Enterobacter* genomes (Supplementary Figure S1), only having a limited degeneracy. These primer sequences covering the *hpaB* and *hpaC* genes were further evaluated in silico by fastPCR [30] analysis. To avoid that the primers would be able to detect fragments within other closely related organisms, the primer sequences were individually tested against all 8,346 non-*Enterobacter* (Supplementary TableS1). The reverse primer hpa_625R is relatively non-specific, with fastPCR matches to all 1,069 *Enterobacter* genome sequences and to 4,326 of the 8,346 (51.8%) non-*Enterobacter* sequences. By contrast, the forward primer hpa_521F had matches in 1,067 (99.8%) of the *Enterobacter* genomes as well as four matches (< 0.1%) in the non-*Enterobacter* genomes, the latter of which all belonged to the species *L. pneumoniae*. The second forward primer hpa_521F had matches in 1,041 (97.4%) *Enterobacter* strains and 16 (0.2%) non-*Enterobacter* taxa, with all of the latter designated as *Kluyvera* or *Klebsiella* species. The genomes designated as *Klebsiella* spp. demonstrated an inconclusive taxonomy check status in the NCBI quality analysis and likely also belong to the genus *Kluyvera*. Overall, it was predicted that only *Enterobacter* strains would yield two amplicons of 110 bp and at 370 bp sizes in a single PCR reaction (Fig. 2; Supplementary Figure S2). The smaller amplicon, hereafter designated as amplicon 1, is expected to occur for *L. pneumoniae*, while the larger amplicon, hereafter amplicon 2, would occur for some *Kluyvera* strains.

#### Evaluation of the primer set against a test set of known species

The designed semi-nested PCR primer set comprising two forward and one reverse primer was tested on a set of 123 strains comprising 66 *Enterobacter* strains belonging to ten distinct species, as well as 57 non-*Enterobacter* taxa belonging to 20 distinct genera (Table 1). All strains were confirmed by MALDI-TOF MS in combination with identification based on the calculated molecular masses of ribosomal proteins by MabritecCentral for their identification. Average nucleotide identities (ANI) to the respective type strains was done for a selection of *Enterobacter* strains (indicated in Supplementary Table S2) to confirm the identification results.

*Enterobacter* strains positive in the PCR expectedly yielded two amplicons 1 and 2, while strains only displaying the amplicon 1 were classified as *L. pneumoniae* (Table 1; Fig. 2; Supplementary Figure S3). Strains showing no or only the amplicon 2 did not belong to the genus *Enterobacter*. No side products were observed in any of the reactions. Primers were also evaluated in a qPCR approach, which might be more convenient than gel visualization for the analysis of many strains. The melting curves for the multiplexed setting were not clearly interpretable due to the presence of non-specific amplicons (Supplementary Figure S4). When the primer sets (hpa_287F and hpa_625R; hpa_521F and hpa_625R) were evaluated separately, clear identification of the *Enterobacter* strains was possible, similar to the conventional PCR approach. If applied in a qPCR approach, it is recommended to screen first with the primer pair hpa_521F and hpa_625R, and in a second step to use hpa_287F and hpa_625R on all positive strains from the first screen.

#### Confirmation of the amplicons and marker gene resolution

To confirm the specificity of the PCR product and evaluate its potential for species identification, amplicon 2 of the PCR reactions (hpa_287F and hpa_625R) was sequenced for eleven of the 123 strains (representing ten distinct *Enterobacter* sp. and *Kluyvera* sp. 9062RM

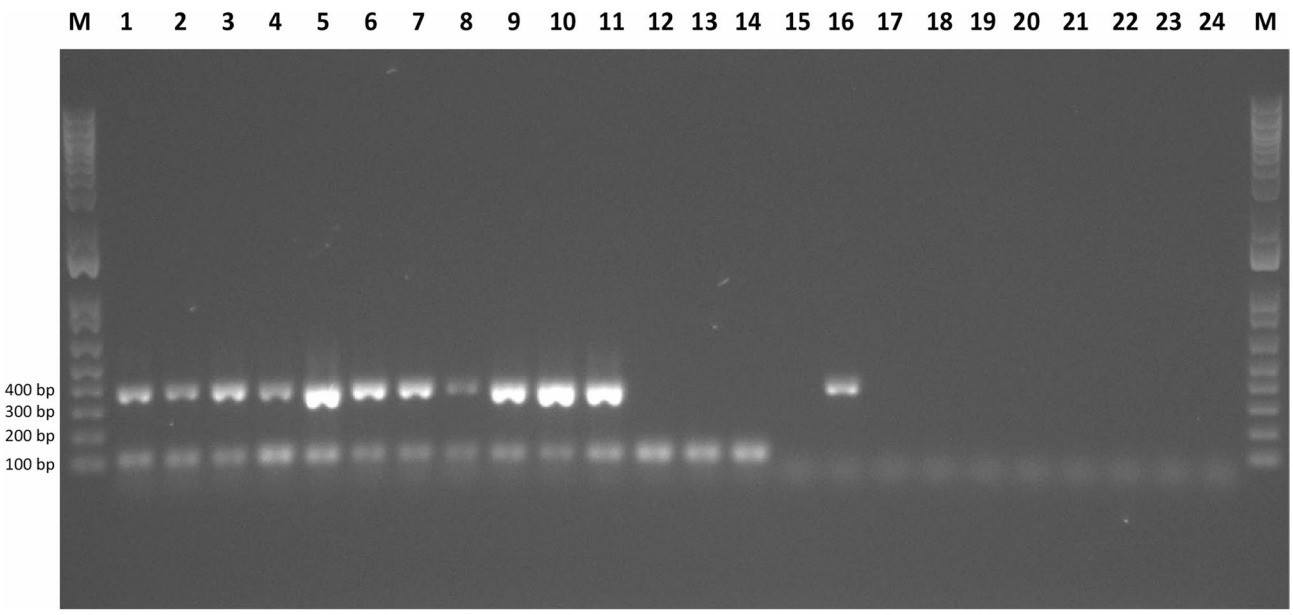


**Fig. 2** Agarose gel electrophoresis of PCR amplicons: (1) *Enterobacter cloacae* ATCC13047$^T$, (2) *Enterobacter cancerogenus* LMG 2693$^T$, (3) *Enterobacter asburiae* DSM17506$^T$, (4) *Enterobacter soli* LF7$^T$, (5) *Enterobacter ludwigii* ECWSU1, (6) *Enterobacter kobei* AZ3a, (7) *Enterobacter mori* 19UT013, (8) *Enterobacter hormaechei* subsp. *steigerwaltii* 20TX0131, (9) *Enterobacter bugandensis* 20TX0138, *Enterobacter roggenkampii* 20CA0095, 11. *Enterobacter wuhouensis* 20GA0343, 12. *Leclercia pneumoniae* LMG 5336, 13. *Leclercia pneumoniae* ATCC 27,994, 14. *Leclercia pneumoniae* ATCC 27,993, 15. *Escherichia coli* SM06, 16. *Kluyvera* sp. 9062RM, 17. *Pseudocitrobacter* sp. CIP 102,343, 18. *Cronobacter sakazakii* ATCC29544$^T$, 19. *Siccibacter turicensis* LMG23730$^T$, 20. *Phytobacter diazotrophicus* ATCC 27,981, 21. *Buttiauxella* sp. LMG 5339, 22. *Pantoea agglomerans* ATCC 27,155$^T$, 23. *Franconibacter pulveris* LMG24057$^T$, 24. negative control; M: 1 Kb Plus DNA Ladder (Invitrogen)

which was positive for amplicon 2) (Supplementary Table S3). The only strain with unclear results was 20GA0343, which was identified as *E. wuhouensis* based on MALDI-TOF MS and ANI. The best BLASTN matches for this strain were *E. huaxiensis* 090008 and *E. wuhouensis* AV1 with a sequence coverage of 99% and 100%, and nucleotide identity of 95.20% and 93.43%, respectively. For the other strains, BLASTN against the *Enterobacter* genomes gave consistent best hits that were in accordance with the MALDI-TOF MS/MabritecCentral identification. The five strains with available genomes all displayed 100% nucleotide identity for the sequenced amplicons with the corresponding genome sequences.

To further evaluate the potential of the *hpaBC* gene fragment sequence for *Enterobacter* species identification, its performance as phylogenetic marker gene was compared with other commonly used marker genes (*dnaJ*, *atpD*, *gyrB*, *infB*, *rpoB* and *hsp60*). Single gene and MLSA trees were generated using the gene sequences derived from the 4,276 publicly available *Enterobacter* spp. genomes. For all single gene trees (including the *hpaBC* gene fragment), individual strains from a certain species clustered with those of other species, while some species were separated in multiple clades, including for the *hpaBC* gene fragment (Fig. 3; Supplementary Figure S5). While there are differences between the marker genes, none of them can clearly separate all species. The *dnaJ* gene, which has been reported to allow precise *Enterobacter* species assignments [31], also does not provide a good resolution for some of the species. A MLSA on the basis of the concatenated *gyrB*, *rpoB*, *infB* and *atpD* gene sequences showed a more accurate resolution, however, it also does not allow clear separation of all species (Fig. 3). For example, *E. asburiae* SD4L was initially identified as *E. cloacae* based on the individual *gyrB*, *rpoB*, *infB* and *atpD* genes, but it was reclassified to *E. asburiae* after the complete genome was sequenced [32, 33].

Despite the limited resolution, sequencing of single or multiple genes may still represent a good approach if more accurate methods are not accessible. Some relevant species, including *E. cloacae*, *E. kobei* and *E. ludwigii*, are clearly separated in all gene trees (Fig. 3, Supplementary Figure S5), including the phylogeny based on the *hpaBC* gene fragment. As such, the *hpaBC* fragment, along with other single marker genes, can be used for preliminary species identification, but the results should be treated with caution.

### Strain identification and PCR validation

The developed PCR was tested on 120 strains (catalogued as "*Enterobacter* spp.") obtained from the USDA-ARS culture collection (NRRL) (Supplementary Table S4). The assay was performed as a blind assay using only lab-internal strain numbers for all strains to avoid influence based on the previous identification.

**Table 1** Overview of PCR results in the test set of known species (detailed list in Supplementary table S2). A + or – sign indicates that all tested stains were positive or negative, respectively. Variable results were not obtained

| Organism | Number of tested strains | Amplicon 1 | Amplicon 2 | PCR Result |
|---|---|---|---|---|
| *Enterobacter asburiae* | 3 | + | + | + |
| *Enterobacter bugandensis* | 2 | + | + | + |
| *Enterobacter cancerogenus* | 2 | + | + | + |
| *Enterobacter cloacae* | 2 | + | + | + |
| *Enterobacter hormaechei* | 12 | + | + | + |
| *Enterobacter kobei* | 4 | + | + | + |
| *Enterobacter ludwigii* | 33 | + | + | + |
| *Enterobacter mori* | 6 | + | + | + |
| *Enterobacter roggenkampii* | 1 | + | + | + |
| *Enterobacter wuhouensis* | 1 | + | + | + |
| *Acinetobacter* | 1 | - | - | - |
| *Buttiauxella* | 1 | - | - | - |
| *Cronobacter* | 9 | - | - | - |
| *Curtobacterium* | 1 | - | - | - |
| *Enterococcus* | 3 | - | - | - |
| *Escherichia* | 1 | - | - | - |
| *Franconibacter* | 5 | - | - | - |
| *Klebsiella* | 2 | - | - | - |
| *Kluyvera* | 1 | - | + | - |
| *Kosakonia* | 10 | - | - | - |
| *Leclercia* | 4 | + | - | - |
| *Lelliottia* | 1 | - | - | - |
| *Micrococcus* | 1 | - | - | - |
| *Pantoea* | 4 | - | - | - |
| *Phytobacter* | 6 | - | - | - |
| *Pseudocitrobacter* | 1 | - | - | - |
| *Pseudomonas* | 1 | - | - | - |
| *Psychrobacter* | 2 | - | - | - |
| *Rahnella* | 1 | - | - | - |
| *Siccibacter* | 2 | - | - | - |

All strains were first identified with MALDI-TOF MS in combination with the MabritecCentral database (Supplementary Figure S6). Of these, 107 could be assigned to the species level, while eleven strains were only assigned at genus level with MALDI-TOF MS. One strain (B-51110) gave low matches with *Kosakonia* and *Phytobacter*, while the other strain (B-455) gave no match to any known species. The latter strain may potentially be a Gram-positive bacterium, for which the direct smear method is not suitable for identification [34]. The identification was confirmed for 23 isolates by WGS data (Supplementary Table S4).

Forty-four of the strains maintained in the NRRL predate the establishment of the genus *Enterobacter* and were thus originally deposited as *Aerobacter*. Furthermore, 15 strains were catalogued as species that are no longer part of the genus, namely *Enterobacter* (*Klebsiella*) *aerogenes*, *Enterobacter* (*Lelliottia*) *nimipressuralis*, *Enterobacter amnigenus* (*Lelliottia amnigena*) and *Enterobacter* (*Kosakonia*) *cowanii*. It was assumed that these strains would give negative results for the *Enterobacter* PCR.

Using the PCR assay designed in this study, 78 of the 120 strains were identified as *Enterobacter*, while no visible amplicon was observed on the agarose gel for the remaining 42 strains. All positive reactions matched with the MALDI-TOF MS identification as belonging to the genus *Enterobacter* (Table 2). These findings were consistent with the expected performance of the designed primer set and suggested its potential utility for *Enterobacter* identification.

Overall, 144 *Enterobacter* strains out of 243 strains were correctly identified with the developed PCR assay, from which 42 *Enterobacter* and 40 non-*Enterobacter* were confirmed on the basis of WGS data, giving a 100% accuracy (Supplementary Table S5). However, it has to be considered that the set of tested strains suffers from sampling bias as many strains are from the same isolation source and therefore some species are overrepresented. Further validation of the PCR is needed, especially for the *Enterobacter* species that were not tested in this study. However, it is still regarded to be trustful due to the large scale of genomic comparisons that were performed for the primer design. As currently undescribed species clades of *Enterobacter* were also included, it is plausible to remain valid even after future taxonomic changes within the genus. It tested negative for a broad range of non-*Enterobacter* strains, including former *Enterobacter* members such as *Kosakonia*, *Leclercia*, and *Lelliottia*, as well as the closely related ESKAPE pathogen *K. pneumoniae* and strains from other genera that possess the target genes including *Escherichia* and *Citrobacter*.

## Conclusions

The novel semi-nested PCR approach provides a reliable, rapid, and cost-effective way to identify *Enterobacter* strains to genus level. Subsequent sequencing of the PCR product allows a preliminary species assignment, but it should be noted that neither the *hpaBC* fragment, nor the other frequently used single copy marker genes individually, provide a perfect resolution for all *Enterobacter* species. The validity of the *Enterobacter*-specific PCR assay was further confirmed by evaluation of a comprehensive collection of potential *Enterobacter* species, where two thirds of NRRL strains ($n = 78$ of 120) listed in the catalogue as *Enterobacter* were now confirmed as belonging to the genus. The PCR assay is therefore suitable for the identification of large sets of putative *Enterobacter* strains and would be especially useful for less equipped laboratories which do not have access to MALDI-TOF MS or genome sequencing platforms.

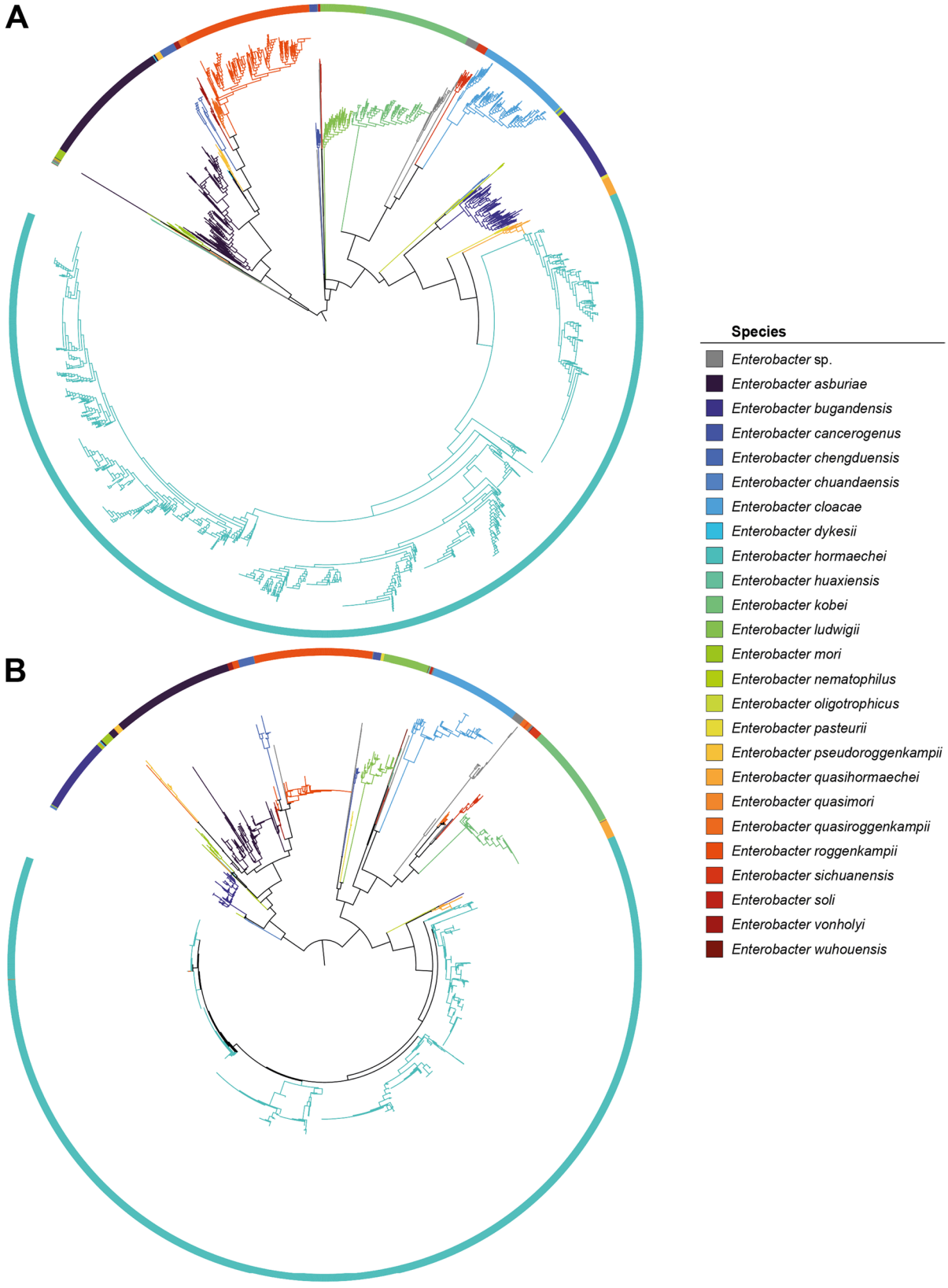


**Fig. 3** Multi-locus sequence analysis (**A**; 10,515 bp) and single gene trees of the *hpaBC* PCR amplicon 2 (**B**; 370 bp) from 4276 genomes representing all species of *Enterobacter*, including clades of currently undescribed species

**Table 2** Overview of PCR results in the validation set (detailed list in Supplementary Table S4). A + or– sign indicates that all tested stains were positive or negative, respectively. Variable results were not obtained

| Organism | Number of tested strains | Amplicon 1 | Amplicon 2 | PCR Result |
|---|---|---|---|---|
| *Enterobacter asburiae* | 19 | + | + | + |
| *Enterobacter bugandensis* | 4 | + | + | + |
| *Enterobacter cancerogenus* | 2 | + | + | + |
| *Enterobacter cloacae* | 2 | + | + | + |
| *Enterobacter hormaechei* | 21 | + | + | + |
| *Enterobacter kobei* | 4 | + | + | + |
| *Enterobacter ludwigii* | 17 | + | + | + |
| *Enterobacter mori* | 4 | + | + | + |
| *Enterobacter pasteurii* | 2 | + | + | + |
| *Enterobacter soli* | 1 | + | + | + |
| *Enterobacter wuhouensis* | 2 | + | + | + |
| *Acinetobacter* | 2 | - | - | - |
| *Cronobacter* | 2 | - | - | - |
| *Enterococcus* | 1 | - | - | - |
| *Hafnia* | 2 | - | - | - |
| *Klebsiella* | 8 | - | - | - |
| *Kosakonia* | 11 | - | - | - |
| *Leclercia* | 1 | - | - | - |
| *Lelliottia* | 4 | - | - | - |
| *Pantoea* | 1 | - | - | - |
| *Pseudescherichia* | 1 | - | - | - |
| *Pseudomonas* | 3 | - | - | - |
| *Raoultella* | 4 | - | - | - |
| *Serratia* | 1 | - | - | - |
| Not identified | 1 | - | - | - |

## Methods

### Bacterial strains

The bacterial strains used in this study are listed in Supplementary Tables S2 and S4. Most of the strains were received as *Enterobacter* from the NRRL culture collection (https://nrrl.ncaur.usda.gov/) and from the Stop-The-Rot project surveys. All strains were streaked on Luria-Bertani (LB) agar and grown for 24–48 h at 28 °C, which is suited for both environmental and clinical isolates [1]. Subsequently, they were stored in 25% (*vol*/*vol*) LB-glycerol stocks at −80 °C.

### Strain identification with MALDI-TOF MS combined with MabritecCentral

All evaluated strains were grown for 24 h on LB plates, and a loopful of cell material from a single colony was smeared onto target slides (Fleximass-SR48) in quadruplicate. The spots were overlaid with 1 µl sinapinic acid (40 mg/mL) matrix solution diluted in 60% acetonitrile and 0.3% trifluoroacetic acid and air-dried for a few minutes at room temperature. On each target plate, the reference strain *Escherichia coli* DH5α was used for system calibration and quality control. The mass spectra were generated using an Axima Performance instrument (Shimadzu, Kyoto, Japan), with detection in the linear positive mode at a laser frequency of 50 Hz and within a mass range 4 to 30 kDa. Peaks were picked from raw spectra as previously described [35]. The generated ascii files were uploaded to MabritecCentral (https://mabriteccentral.com) for species identification.

### Target gene selection

Genome sequences were obtained from the National Center for Biotechnology Information (NCBI) database [36]. The type strain genomes of 29 species and subspecies of *Enterobacter* and a selection of 60 genomes of type strains covering all recognized genera of the family *Enterobacteriaceae* were used for initial comparative genomics. A tree based on 81 bacterial core genes conserved among all 89 genomes was created with UBCG2 v2.0 [28] and visualized with iTOL 6.9.1 [37]. Orthofinder v2.5.4 [29] was used with default parameters to identify homologous genes that are abundant in all *Enterobacter* strains but lacking in most other *Enterobacteriaceae* [29].

All 4,413 available RefSeq assemblies assigned as *Enterobacter* were downloaded from the NCBI database on 23 September 2022. The completeness of these genomes was evaluated with BUSCO v5.4.3 [38]. Genome assemblies displaying < 97% BUSCO completeness were excluded for the primer design. In order to confirm the taxonomic assignment of the remaining 4,294 *Enterobacter* strains, their genomes were compared against the type strain genome dataset using fastANI v1.3 [39]. Eighteen of these genomes were not *Enterobacter* according to the ANI comparisons and therefore excluded from the selection. BLASTN v2.10.0 was used to confirm whether the genomes of the 4,276 *Enterobacter* strains contained the target gene.

### Primer design

To reduce redundancy, a subset of 1,069 assemblies of the remaining 4,276 *Enterobacter* spp. was created by selecting only one strain per species for each NCBI BioProject. The sequences of the target gene *hpaB* (with the neighboring genes *hpaA* and *hpaC*) of this subset were used for primer design. A multiple sequence alignment (MSA) of the target sequence was generated with MUSCLE v3.8.1551 [40] and Degeprime v1.1.0 [41] was used to detect potential primer sequences. All potential primers that had an exact match with > 99% of the *Enterobacter* sequences were further tested in silico with fastPCR v6.9 [30] (with 0 allowed mismatches at the 3′ end) on all *Enterobacteriaceae* type strain genomes that contain the target gene. As no perfect primer pair could be identified, a second forward primer was designed based on the MSA that should exclude *Leclercia pneumoniae*.

The final primers were tested again in silico on all 8,346 non-*Enterobacter* sequences that were identified with BLASTN using the *hpaBC* region from *E. cloacae* ATCC 13,047$^T$ (CP001918.1 region 743,259 to 745,351) as query sequence against NCBI nucleotide collection (nr/nt) excluding *Enterobacter* (taxid 547) on 06 June 2024.

### PCR test and validation

All evaluated strains were grown on plates as described above and a loopful of cell material was resuspended in ultrapure water and boiled for 10 min at 95°C. The boiled cells were diluted tenfold and directly used as DNA template for the PCR. The primers hpa_287F (5'-GGCGCGCCATGATCTCY-3'), hpa_521F (5'-GCTGCRYTATTTCAGCAGCT-3') and hpa_625R (5'-ATGGTCGACCGCTGYATGTC-3') were ordered from Microsynth AG (Balgach, Switzerland).

The PCR mix for one reaction contained 4 µl KAPA2G Robust 2× mix, 0.4 µl hpa_287F (10µM), 0.2 µl hpa_521F (10µM), 0.4 µl hpa_625R (10µM), 1 µl diluted boiled cells and 2 µl nuclease-free water in a total volume of 8 µl. PCR amplification was performed in a T100 thermal cycler (Bio-Rad, Hercules, USA) with reaction conditions including an initial denaturation step at 95 °C for 3 min, followed by 30 cycles off amplification (95 °C for 15 s, 62 °C for 15 s, and 72 °C for 15 s) and a final extension step at 72 °C for 1 min.

Quantitative PCR (qPCR) was performed in a Light Cycler®480 II (Roche, Basel, Switzerland). One reaction mix contained 5 µl LightCycler 480 SYBR Green I Master mix, 0.5 µl of each primer (10µM), 2 µl diluted boiled cells and 1.5–2 µl nuclease-free water in a total volume of 10 µl. Cycling conditions were as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 62 °C for 15 s and 72 °C for 15 s. A melting curve was performed after the amplification (raising 1 °C per second, from 65 °C to 97 °C).

Sanger sequencing.

PCR products were cleaned up with the NucleoSpin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) and sent to Microsynth AG for Sanger sequencing with both the forward primer hpa_287F and reverse primer hpa_625R. The obtained sequences were trimmed and curated using UGENE v50.1 [42]. The consensus sequences were compared against the NCBI nucleotide collection (nr/nt) using BLASTN.

### Single gene trees and MLSA

The full sequences of the marker genes *atpD*, *dnaJ*, *gyrB*, *infB*, *rpoB* and *hsp*60 as well the sequence of the *hpaBC* long amplicon (370 bp) were extracted from the 4,276 *Enterobacter* spp. genomes using BLASTN with the corresponding sequences from *E. cloacae* ATCC 13,047$^T$ (NC_014121.1; locustags: ECL_RS00020, ECL_RS01185, ECL_RS22690 ECL_RS25685, ECL_RS03995, ECL_RS02580). Each of the genes was missing in 1–2 genomes and incomplete in 0–2 genomes and these were therefore excluded from the respective trees. Multiple sequence alignments of each gene fragment were generated with MUSCLE v3.8.1551 [40]. For MLSA, the alignments of *atpD*, *gyrB*, *infB* and *rpoB* were concatenated (totalizing 10,515 bp). All phylogenies were created with fasttree v2.1 [43] and visualized with iTOL 6.9.1 [37, 44].

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04175-1.

Supplementary Material 1.

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### Authors' contributions

SJ, TAC and THMS designed the study. SJ and JP performed experiments and analyses. SJ and THMS wrote the manuscript text. SJ prepared figures and tables. All authors read and approved the final manuscript.

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### Data availability

Data generated or analyzed during this study are included in this published article and its supplementary information files. The MALDI-TOF MS data are provided in the Zenodo archive https://doi.org/10.5281/zenodo.13992849 and will be made publicly available on acceptance of the manuscript.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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