OPEN ACCESS International Journal of Applied Biology



ISSN : 2580-2410
eISSN : 2580-2119

# The Role of Molecular Systematics in Microbiological Research and Public Health: A Systematic Review

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## Abstract

The robust development of molecular biology and bioinformatics in recent years has given researchers vital tools for addressing long-standing issues in all branches of biology. The process of reconstructing phylogenetic relationships using molecular data is known as "molecular systematics." In this systematic review, the utilization of molecular techniques in systematics was described in the two fields of microbiological research and public health. Twenty articles were included in this study, showcasing nine unique molecular methods with distinct advantages and limitations of use. It was revealed that most articles under biological research aim to identify a specific group of microorganisms. At the same time, the determination of phylogenetic relationships and outbreak investigation were the main goals of public health utilization of molecular systematics techniques.

**Article History**
Received May 25, 2023
Accepted June 19, 2023

**Keyword**
*Molecular Systematics; Public Health,; Bioinformatics; Microbiology.*

## Introduction

Hundreds of millions of people worldwide are now afflicted with illnesses caused by bacteria, viruses, protists, and fungi. According to the most recent study from the WHO (2022), people are anticipated to live longer and enjoy better health for an increasing number of years. The global healthy life expectancy (HALE) improved from 58.3 to 63.7 years. Despite this, health disparities continue to take a disproportionate toll on the lives and health of those in areas with fewer resources. The bulk of communicable illnesses, including HIV, tuberculosis (TB), neglected tropical diseases, malaria, hepatitis B, and COVID-19, continue to be borne by low- and middle-income nations. Despite substantial expenditures and advancements in communicable disease initiatives, these nations continue to shoulder most of this burden. Without systematics, which identifies species and explores their evolutionary links, it would be hard to make significant progress against these illnesses. It is improbable that public health

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initiatives will be successful if they do not target a particular condition. Due to evolution, many species' anatomy, biochemistry, and DNA have become more distinct. Creatures belonging to one clade may be able.

To cause human illnesses, microbes may not belong to some other, closely related clade. In order to correctly identify the underlying cause of disease and study the processes that contribute to its development, it is crucial to have a thorough understanding of the differences within clades.

Given that just 10–20 percent of species are estimated to have been described, it should not surprise that new disease-causing agents are continuously being identified. In addition, the advent of new techniques has shown that single species of pathogenic organisms sometimes consist of two or more different species. Pathogenicity, treatment sensitivity, and reaction to the formulation of a vaccine, for instance, cryptic species, may differ significantly from one another. Previously thought of as innocuous microorganisms are now capable of causing sickness in immunocompromised populations, which are rising at an alarming pace. Genetic mutations are the underlying cause of the continuing alteration in the genetic composition of organisms. Changes in pathogenicity are thus feasible and may be frequent. An increase in the use of chemicals for disease prevention results in the evolution of chemical resistance and the number of genetically different strains of a pathogenic taxon.

This article discussed the relevance of molecular systematics in microbiological research and public health by describing several molecular systematic methodologies using genome and non-genome sequencing in microbiology research. In addition, the advantages and limitations of these techniques were also discussed. This article also provided insights on the current status of molecular systematics in the country from the pillar institutions of public health in the country.

## Materials and Methods

### Literature Search Strategy

Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed in this review. A total of forty-two (42) full-text studies were collected from various databases, including PubMed, ResearchGate, ScienceDirect, Google Scholar, Oxford Academic, Wiley Online, and EBSCOhost. The search terms used were as follows: public health, microbiology, systematics, research, and molecular biology.

### Eligibility Criteria

The titles and summaries of the articles in molecular biology, health sciences, and education were reviewed to include peer-reviewed experimental research articles, literature reviews, and synthesis articles which demonstrated the importance and use of molecular techniques in microbial systematics. Only full-text articles written in English and published between 2012 to 2022 were included in this review to ensure updated information on the research topic.

### Data Extraction

Studies selected from databases used were then imported to Microsoft Excel and were tabulated according to the author, year of publication, study objectives, the molecular technique used, organism involved, advantages and limitations of the technique, and its impact on microbial systematics.

### Study Selection Flowchart

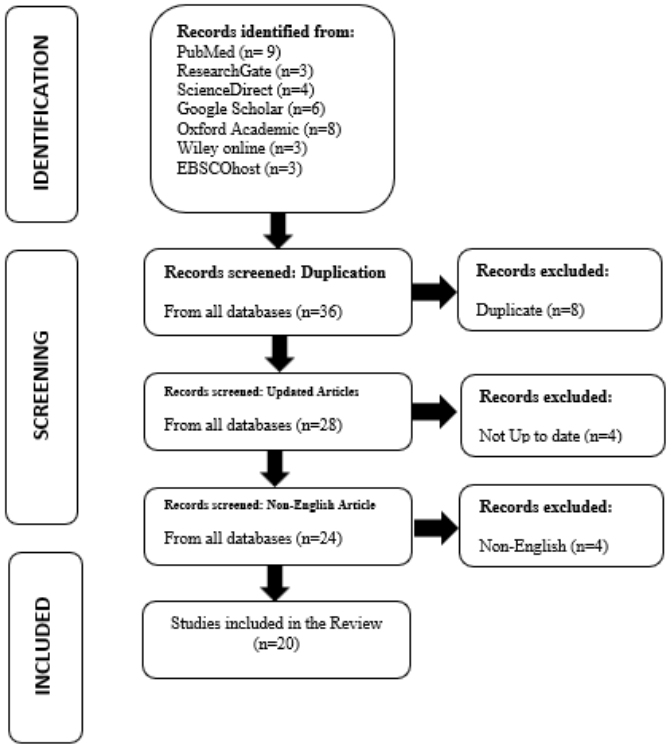


**Figure 1.** Study Selection Flowchart

## Results

A total of thirty-six full-text research articles were collected from various databases, books, and websites. During the screening, eight duplicate studies were removed. Another four studies were excluded since it was published before 2012, and four were also removed since the articles were not written in English. Overall, there were twenty research articles analyzed in this systematic review.

This review was able to document nine molecular techniques, including 1) 16S rRNA gene-based ribotyping/ oligotyping; 2) amplified ribosomal DNA restriction analysis (ARDRA), 3) random.

**Table 1.** Summary of Research Articles (n=20) Analyzed in The Systematic Review Indicating Its Role in Biological Research and Public Health.

| Author and Year of Publication (2012-2022) | Role of Molecular Systematic Microbiology (Identification/ Determination of Phylogenetic Relationship) | Microorganism Involved | Molecular Technique Used | Cited Advantages of the Molecular Technique | Cited Limitations of the Molecular Technique | Impact on Microbial Systematics |
|---|---|---|---|---|---|---|
| | | | **MICROBIOLOGICAL RESEARCH** | | | |
| **Nunez et al. (2017)** | Determination of phylogenetic relationship among *Acidithiobacilli* species complex | *Acidithiobacillus* spp. | 16S rRNA gene-based ribotyping, oligotyping, and multilocus sequencing analysis (MLSA) | Oligotyping was used to profile high entropy nucleotide positions and resolve meaningful differences between closely related trains at the 16S rRNA gene level. | Due to oligotyping greater discriminatory power, MLSA was used as a proxy for genome-wide divergence in a smaller but representative set of strains. | At least six new lineages or phylotypes, supported by the different methods used in this study, were evident within the *acidithiobacillus* species complex and were determined. |
| **Jarocki et al. (2016)** | Identification of the members of the *Bifidobacterium* genus | *Bifidobacterium* spp. | ARDRA, RAPD-PCR, rep-PCR, and SDS-PAGE fingerprinting | Highly discriminatory, easy-to-handle, and relatively low-cost procedure for rapid differentiation of *Bifidobacteria* at the intra-species level. | Appears to be more laborious, and time-consuming compared to other PCR-based methods | Rapid and accurate identification of these microorganisms at the species, subspecies, and even strain level due to the increasing application of *Bifidobacteria* |
| **Hwang et al. (2022)** | For inferring phylogenetic relationships and evolutionary study | Epilithic diatoms | RAPD-PCR | Small subunit (SSU) rRNA coding gene is the most widely used and suitable for inferring phylogenetic relationships, and the ribulose-1,5bisphosphate carboxylase/oxygenase large subunit (rbcL) gene appears more suitable for evolutionary study. | Not mentioned in the article | Identification of three new species of diatoms |
| **Gomila et al. (2017)** | Identification of *Pseudomonas syringae* species | *Pseudomonas syringae* species group | Three-Gene Multilocus Sequence Analysis (3-Gene MLSA) and Whole-Genome Comparisons | This study was able to circumscribe the *P. syringae* species complex and classify its strains into species according to the taxonomic rules and thresholds. | Not mentioned in the article | Classification of *Pseudomonas* isolates |

| Author and Year of Publication (2012-2022) | Role of Molecular Systematic Microbiology (Identification/ Determination of Phylogenetic Relationship) | Microorganism Involved | Molecular Technique Used | Cited Advantages of the Molecular Technique | Cited Limitations of the Molecular Technique | Impact on Microbial Systematics |
|---|---|---|---|---|---|---|
| **Fang et al. (2019)** | Identification of *Shewanella*-type strains | *Shewanella* type strains | Standard multilocus sequence analysis (MLSA) | Allows researchers to make rapid, economic, and precise identification of *Shewanella* strains | Not mentioned in the article | *Other strains of Shewanella* were also discovered in food samples consisting of both marine products and cooked food for sale. |
| **Klaus et al. (2022)** | Identification of Hyperthermophilic Ehaeon *Thermococcus* sp. Strain | Hyperthermophilic Ehaeon *Thermococcus* sp. Strain | Activity-Based Protein Profiling | Allows researchers to make rapid, economic, and precise identification of Thermococcus strains | Not mentioned in the article | ABPP, in extremophilic environments, represents an alternative approach to addressing biological diversity and screening for novel biocatalysts. |
| **Babafemi et al. (2017)** | Detection of *Mycobacterium tuberculosis* in pathological samples: | *Mycobacterium tuberculosis* | Real-time polymerase chain reaction (RT-PCR) based systems | RT-PCR assay demonstrated a high degree of sensitivity for pulmonary TB and good sensitivity for extra-pulmonary TB. | Not mentioned in the article | It indicated a high degree of specificity for ruling in TB infection from sampling regimes. |
| **Ma (2022)** | Identification of coronavirus | Coronavirus | Isothermal amplification technologies | Eliminating the need for expensive equipment such as thermocyclers. | conducted at a constant temperature, | Has mild reaction conditions and low instrument dependence. |
| **Coertse et al. (2019)** | Identification of rabies virus | Rabies virus | Reverse transcription recombinase polymerase amplification assay | allowed for very quick and sensitive detection of rabies virus | Do not cover the full genetic diversity of RABV | Considered as a supplementary tool where basic laboratory infrastructure is available, |
| **Wang et al. (2015)** | Identification of Listeria monocytogenes | Listeria monocytogenes | Multiple Inner Primers-Loop-Mediated Isothermal Amplification (MIP-LAMP) | Accuracy was shown to be 100% when compared to the culture-biotechnical method. | May not detect extremely low levels of microorganisms that are non-culturable cells | MIP-LAMP methodology was demonstrated to be a reliable, sensitive, and specific tool for rapid detection of *L. monocytogenes* strains. |
| | | | **PUBLIC HEALTH** | | | |
| **Srinivasan et al. (2015)** | Identification of most common hospital-associated bacterial pathogens as well as | *Acinetobacter baumanni; Bordetella pertussis; Burkholderia* | Non-16S rRNA-based molecular identification using Naïve | Non-16S-based identity produced high confidence genus level identification and | Discordances between clinical and 16S-based identities due | wide variety of bacterial species that are relevant to clinical settings can be accurately |

| Author and Year of Publication (2012-2022) | Role of Molecular Systematic Microbiology (Identification/ Determination of Phylogenetic Relationship) | Microorganism Involved | Molecular Technique Used | Cited Advantages of the Molecular Technique | Cited Limitations of the Molecular Technique | Impact on Microbial Systematics |
|---|---|---|---|---|---|---|
| | endemic community-acquire | *cenocepacia; Burkholderia cepacia; Burkholderia dolosa; Burkholderiamultivorans; pyrrocinia; Burkholderiastabilis; vietnamiensis; Citrobacter freundii; Enterobacter aerogenes;* | Bayes Classifier and an alignment-based approach | good species level identification | to insufficient representation of the clinical identities, phenotypic misidentification, new taxonomic or phylogenetic placements | identified routinely by microbiologists using high-quality sequences produced from 16S rRNA gene amplicons. |
| **Banoon et al. (2019).** | Characterization and construction of phylogenetic relationship of different isolates of bacterial species from different sources in Babylon Province hospitals | Staphylococcus aureus | Random Amplified Polymorphic DNA (RAPD) Fingerprinting Technique | RAPD markers proved to have fingerprinting and diagnostic potential | The technique cannot suggest the possible and frequent occurrence of mutants in S. aureus in different host cells | The DNA signature assigned to each S. aureus for epidemiological studies, medical diagnostics, and the location of novel pathogenic strains of bacteria is helpful. |
| **Dallal et al. (2016).** | Identification of *S. aureus* subtypes isolated from food samples | *Staphylococcus aureus* | PCR-based coagulase genotyping by RFLP analysis (coa-RFLP) | Classification based on the RFLP of the coa gene is a simple and accurate method for typing *S. aureus* isolated from various sources. | Not mentioned in the article | With the aid of this typing approach, phylogenetic relationships or lineages between isolates from various sources can be established. |
| **Meier-Kolthoff et al. (2014)** | Characterization of the type of strain (U5/41T) of *Escherichia coli* for subspecies delineation | *Escherichia coli* | DNA: DNA-hybridization (dDDH | It yields groups with a similar upper bound of character divergence. It directly addresses how to best cluster the sequences of genomes of interest. | Inconsistencies can occur when distance or similarity thresholds are used and the underlying distances specifically deviate from ultrametricity. | Analyses of the genome sequences of a large number of E. coli strains, which in turn suggests the presence of five subspecies within E. coli. |
| **Ashton et al. (2016)** | Characterization of Salmonella for routine public health surveillance | Salmonella spp. | Multilocus sequence typing (MLST) | The designation accurately depicts the relatedness of the strains, and this method is automatable. | In the MLST database, a number of STs (including subspecies II–IV) lacked a serovar | The true phylogenetic relationship between isolates can be revealed using MLST data. Serotypes can |

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|---|---|---|---|---|---|---|
| | | | | | classification, and a surprisingly high number of unique STs were discovered. | correctly be assigned to monophasic strains with imperfect antigenic structures. |
| **Masim et al. (2021)** | Identification of Methicillin Resistant *Staphylococcus aureus* and Outbreak Investigation | Methicillin-Resistant *Staphylococcus aureus* | Whole Genome Sequencing, Multilocus sequence typing (MLST) | The entire genome is scrutinized, and in one test, every single variant in the genome is identified; AMR and virulence genes were captured either chromosomal or acquired | Requires high-end equipment and technicians required knowledge and skills in bioinformatics | Genotypic characterization of circulating MRSA strains, with phenotypic and epidemiological data, led to the identification of several global epidemic clones present in the Philippines |
| **Jamoralin et al. (2021)** | Identification of Neisseria gonorrhea | Neisseria gonorrhea | Whole Genome Sequencing, Multilocus sequence typing (MLST) | The entire genome is scrutinized, and in one test, every single variant in the genome is identified; AMR and virulence genes were captured either chromosomal or acquired | Requires high-end equipment and technicians required knowledge and skills in bioinformatics | Genotypic characterization of circulating N. gonorrhea strains, with phenotypic and epidemiological data, led to the identification of several global epidemic clones present in the Philippines |
| **Chilam et al. (2021)** | Identification of *Pseudomonas aeruginosa* and Outbreak Investigation | *Pseudomonas aeruginosa* | Whole Genome Sequencing, Multilocus sequence typing (MLST) | The entire genome is scrutinized, and in one test, every single variant in the genome is identified; AMR and virulence genes were captured either chromosomal or acquired | Requires high-end equipment and technicians required knowledge and skills in bioinformatics | Genotypic characterization of circulating P. aeruginosa strains, with phenotypic and epidemiological data, led to the identification of several global epidemic clones present in the Philippines |
| **Chilam et al. (2021)** | Identification of *Acinetobacter baumannii* and Outbreak Investigation | *Acinetobacter baumannii* | Whole Genome Sequencing, Multilocus sequence typing (MLST) | The entire genome is scrutinized, and in one test, every single variant in the genome is identified; AMR and virulence genes were | Requires high-end equipment and technicians required knowledge and | Genotypic characterization of circulating *Acinetobacter baumannii* strains, with phenotypic |

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|---|---|---|---|---|---|---|
| | | | | captured either chromosomal or acquired | skills in bioinformatics | and epidemiological data, led to the identification of several global epidemic clones present in the Philippines |
| **Lagrada et al (2022)** | Identification of *Salmonella* sp. and Outbreak Investigation | *Salmonella* sp. | Whole Genome Sequencing, Multilocus sequence typing (MLST) | The entire genome is scrutinized, and in one test, every single variant in the genome is identified; AMR and virulence genes were captured either chromosomal or acquired | Requires high-end equipment and technicians required knowledge and skills in bioinformatics | Genotypic characterization of circulating *Salmonella* strains, with phenotypic and epidemiological data, led to the identification of several global epidemic clones present in the Philippines |

Amplified polymorphic DNA polymerase chain reaction (RAPD-PCR); 4) Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); 5) three gene multilocus sequence analysis (3-gene MLSA); 6) genotyping by RFLP analysis; 7) DNA hybridization; 8) multilocus sequence typing (MLST) and 9) whole genome sequencing (WGS). Most of the studies involved in this review utilized these molecular techniques primarily for identification (n=10), determination of phylogenetic relationship (n=5), and outbreak investigation (n=5) (Table 1). It can be noted that the aim of most articles under biological research is to identify a certain group of microorganisms, while the determination of phylogenetic relationship and outbreak investigation were the main goals of public health utilization of molecular systematics techniques.

## Discussion

### Microbial Systematics in the Era of Molecular Biology

One of the many fields of systematics is molecular systematics which makes use of the information contained in molecular data to reconstruct phylogenetic relationships (Mauro and Agorreta, 2010). Systematics plays an important role in the field of biology by providing means for describing the organisms' characteristics and how they can be grouped together. Thus, the two main goals of systematics in microbiology include the identification and analysis of relatedness among microorganisms. In phylogeny, the pattern of historical relationships among lineages of elements can provide an understanding of the groups sharing common

ancestry. Historically, the physical characteristics of organisms and classifying species were according to the most common traits they held. Unfortunately, this method of identification of microorganisms, plants, and animals assumes that it is because they have common physical traits and that they have a common ancestry. It is, therefore, a challenge for taxonomists to begin using molecular differences to compare species. Some morphological characters, such as those based on the shape of a structure, can be less easy to distinguish because of overlaps between different character states (Brown, 2002). On the other hand, in molecular systematics, one can determine the similarities and differences among organisms using variations in protein and deoxyribonucleic acid (DNA) molecules. For instance, some sources of molecular characteristics that can be used, such as a nuclear genome, nuclear ribosomal DNA, mitochondrial genome, and chloroplast genome (Onarici & Sumer, 2002), can provide a much more accurate taxonomic picture of the relationship among microorganisms. The goal of systematic studies is to provide insight into the history of groups of organisms and the evolutionary processes that create diversity among species. In particular, testing of systematic questions and hypotheses, and the estimation of evolutionary processes and patterns, which includes divergence among taxa, can be provided by the study of molecular systematics. Molecular systematics allows the examination of how species have changed over evolutionary time as well as the relationships between species that have no common physical characteristics. Moreover, molecular changes can be used to explore and understand how populations are related evolutionarily and genetically. Specifically, phylogenetics conveys the degree of changes in the evolutionary lines (Hofling et al., 1997).

Molecular systematics requires phylogenetic analysis as a tool for studying phylogenetic relationships of living organisms. For example, the use of molecular markers allows the detection of variations or polymorphisms that exist among individuals in the population for specific regions of DNA. The development of molecular markers has led to an explosion of studies that have used them to answer questions ranging from relatedness among species to the evolutionary history of populations, the amount of genetic variation within a species, patterns of behavior, how patterns of gene expression can vary among closely related populations, and many other aspects of organismal variation (Monsen-Collar & Dolcemascolo, 2010). The nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) that have been most commonly used for phylogenetic research are highly conserved molecular markers and/or gene regions that are useful for investigating phylogenetic relationships at higher categorical levels (Hwang & Kim, 1999). A review by Bhandari et al. (2014), which focuses on two different types of molecular markers, presented a successful understanding of the evolutionary relationships among prokaryotes. Furthermore, the molecular markers can provide the identification of different prokaryotic taxa ranging from phyla to genera in clear molecular terms, and evolutionary relationships among them can also be reliably taken. The availability of fast DNA sequencing techniques, along with the development of robust statistical analysis methods, provided new momentum in the field of molecular systematics.

## Molecular Techniques in the Field of Microbial Systematics

Microbial systematics plays a crucial role in determining the extent of microbial identification and classification applied in numerous research endeavors (i.e., bioremediation). Microorganisms are vastly distributed in nature, but inadequate information about their abundance and scope of diversity exists – creating a huge challenge among microbiologists and other biology experts (Sangal et al., 2016). However, as technological advancements pave the way for these challenges to be addressed, so is a growing knowledge as well as gaps about microorganisms, including their appropriate identification and classification. Many researchers have embraced and advocated for the use of genomics in microbial systematics. For instance, Thompson et al. (2015) reiterated that there is a necessity to establish genome-based microbial taxonomy using complete genome sequences. Accordingly, ecological and clinical microbiology concerns can be addressed from this molecular approach to microbial identification and classification.

There is a growing utilization of 'omics'-based approaches in systematics. However, limitations can ensue when these techniques fail to prioritize major regulatory pathways (Keller et al., 2020), i.e., enzymes involved in microbial pathogen-host interactions. Chemical techniques are introduced to overcome these limitations, particularly in microbial systems. Previously restricted to morphological and biochemical culturing tests alone, the identification and classification of microorganisms can now be carried out utilizing a number of instrumental techniques that have improved efficiency and analytical accuracy. In this section, four molecular approaches in microbial systematics will be described in terms of their application/principle and their advantages and disadvantages over other molecular techniques.

### Activity-Based Protein Profiling (ABPP)

Using chemical probes, this technique directly reads out enzyme activity which is critical in enriching relevant target enzymes in complex and dynamic but regulated systems. This technique targets enzyme identification using its active site for chemical alteration, providing druggable targets for potential minute particle therapeutics (Bachovchin & Cravatta, 2012).

ABPP holds promising benefits in microbial systematics. Used as a tool for the dynamic profiling of multiple enzymes in an endogenous setting (Bachovchin et al., 2010), enzymes that are acquiescent to variation using small-scale particles can be selected. This will, in turn, provide an avenue to identify and filter out discriminatory inhibitors, as well as enzymes with unknown functions and substrates (Niphakis & Cravatt, 2014). In a rich community of bacteria, such as the gut, ABPP positions are advantageous. Mayers et al. (2017) successfully enhanced a complicated sample for relevant but rare enzymes.

Our knowledge of enzyme activity has been enhanced by ABPP. Most research has used some fundamental techniques, such as click chemistry-ABPP (CC-ABPP) and competitive-ABPP strategies, to increase the specificity and accuracy of this technology. But these techniques also hold limitations. For example, nonspecific binding in competitive-ABPP

strategy is a major limitation for activity-based protein profiling. Even at higher concentrations, electrophilic and photoreactive probes would very certainly label proteins in some unspecific ways (Wright & Sieber, 2016). Limitations of bulky protein groups and cell permeability of the probes also exist. This concern has been addressed through click chemistry-ABPP by using a particular probe that can be differentiated with a diversity of identifiers.

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Recognized as one of the most effective and adaptable methods for bacterial protein separation and resolution, SDS-PAGE may be utilized to identify phenotypic diversity and relationships within species (Franco-Duarte et al., 2019). This method involves loading protein samples onto polyacrylamide gel with pores such that the protein can move through the pores in reaction to an electric field. SDS-PAGE can provide information on protein quantity, size, and subunit composition, as well as purify samples for additional analysis (Gallagher, 2012). With an upper stacking gel and a lower resolving gel that have various pH levels and polyacrylamide concentrations, the SDS-PAGE system can be described as a fragmented gel. Proteins can move swiftly and pile into a tight band in the upper stacking gel owing to its lower polyacrylamide content before moving into the higher polyacrylamide-resolving gel for segregation. Using their respective mobilities, the distribution of protein bands along the gel is transformed into numerical values. Through the use of specialized software, these values produce a binary data matrix that provides a similarity matrix, which is then transformed into similarity phenograms (Deosthali et al., 2021).

Particularly for the investigations of the classification of biodiversity among microorganisms, proteins have a great deal of promise for typing strains of clinical relevance and for taxonomic purposesFor instance, cellulase was extracted, screened for, and purified from bacteria found in molasses by Islam and Roy (2018), who also used morphological and biochemical techniques to identify the microorganisms. Using SDS-PAGE, protein molecular weight was identified, which helped in the identification of three bacterial strains. SDS-PAGE is a powerful system that divides protein molecules exclusively on the basis of mass. This method allows for the better classification of several microbial entities.

SDS-PAGE is frequently used to separate complicated protein mixtures with great resolution. But the purposeful denaturation of proteins before electrophoresis is an obvious downside of SDS-PAGE. Enzymatic activity, protein reactions, and other molecular processes are typically impossible to measure on proteins purified by SDS-PAGE. To isolate native proteins for studies of structure-function connections, alternative techniques must be utilized. Functional characteristics, such as the existence of non-covalently metal ions, are also lost.

### Random Amplified Polymorphism of DNA (RAPD) Markers.

Random Amplified Polymorphism of DNA (RAPD) *Markers* is a technique that is utilized to identify DNA-based polymorphism that typically determines the value of DNA-based markers. RAPD markers are a development of employing markers based on Polymerase Chain Reaction (PCR). The standard RAPD method uses PCR to amplify nanogram quantities of whole genomic DNA at low annealing temperatures using short artificial oligonucleotides of random values as primers. Typically, agarose gels are used to separate amplification products before staining them with ethidium bromide.

The detection or classification of several bacterial species has been made possible by markers based on RAPD fingerprints. Without the need for replicating, scanning, or any other type of molecular characterization of the DNA of the target species, RAPD analysis acquires a vast range of genetic identifiers that only require modest amounts of DNA. Due to its speed and user-friendliness, RAPD has been widely employed to fingerprint the microbial population (Panigrahi, 2019). In microbiology, this technique has been used to identify and analyze genetic variation in bacterial species isolated from different sources. For example, Bannon et al. (2019) characterized *Staphylococcus aureus* isolates via RAPD analysis, each of which gives a different genetic profile. Constructing evolutionary relationships using 8 RAPD markers, the entire collection of *S. aureus* isolates was categorized into one main group and nineteen subgroups.

Studies involving related species, as well as analyses at the individual level, have both used RAPD markers. But like any other molecular technique, this approach comes with a downside. Because RAPDs are so sensitive to the reaction conditions, their main setback is their limited reproducibility; hence, highly standardized experimentation procedures are required. Because small random primers are utilized, which have the ability to amplify DNA fragments from a range of organisms, caution must be taken to prevent DNA samples from becoming contaminated during RAPD investigations, which often call for pure, high molecular-weight DNA.

### Restriction Fragment Length Polymorphism (RFLP) Markers

Acting as a molecular identifier unique to a particular clone/restriction enzyme combination, this technique is frequently used to verify the little but noticeable variations in a string of double-stranded DNA. The underlying principle behind the method is the enzymatic digestion of the DNA by endonucleases, coupled by electrophoresis in an agarose gel and Southern hybridization. Electrophoretic tracks resulting from enzymatic digestion can be seen at various spots throughout the length of the genome as restriction enzymes split the DNA into many fragments. Segments of the radioactively tagged DNA may combine with homologous sections of the membrane-immobilized DNA to form hybrids. Once the excess probe that did not undergo hybridization has been removed, exposing the surface to a radiography film will sensitize the film in the areas where the probe settles, producing bands that could be indicative of genetic variation. Using the RFLP marker, the gene sequence of the area that codes for genetic products can be used to identify genetic variation (Mittal, 2013).

Hence, a restriction endonuclease will produce DNA fragments of varying lengths depending on the species and the particular organism.

Being the first DNA-based biomarkers for making genetic linkage maps, the number of RFLP markers is almost limitless and is higher in allelic polymorphism. When combined with other techniques, such as PCR, RFLP allows for increased receptivity and applicability, especially in microbial diversity investigations. Yang et al. (2013) enumerated some of the advantages of this technique when coupled with other molecular methods, including high reliability, co-dominance, and selective neutrality. Because established restriction enzymes are used to create RFLP from particular locations, the results are consistent throughout time and space. For example, successful investigations of the diversity of *V. cholerae* strains (Chowdhury et al., 2010), *Y. pestis* taxonomy (Qi et al., 2016), as well as *S. aureus* coagulase gene diversity (Dallal et al., 2016) have been reported. Moreover, RFLP has also been utilized in phylogenetic studies of microbes to establish phylogenetic characterization, such as the establishment of genetic relationships among clinical strains of *Aspergillus fumigatus* and species identification of methicillin-resistant *Staphylococci* using mitochondrial DNA regions (Mittal, 2013).

RFLP has the benefit of being a rapid, easy, and precise molecular technique for population profiling and identification. However, it has less discriminatory strength and costs more to run than many other fingerprinting methods, such as RAPD (Tabit, 2016). Hence, this technique requires a lot of work and time. Furthermore, this method can only examine particular mutations at enzyme cut sites, which restricts the ability to identify whole genome variation. Finally, the applicability of RFLP markers is constrained by the relatively low polymorphism and requirement for radioisotope detection (Yang et al. 2013).

Numerous molecular methods, some of which are presented here, enable the identification and characterization of microorganisms and establish different levels of subspecific and interspecies similarity. From the standpoint of microbial systematics, it is notable that a single approach might not provide all of the necessary information. To determine the levels of similarity rather accurately among microorganisms, it is essential to use many techniques.

## Genome Sequencing Unfolds the Genetic Relationship Among Microorganisms

A unique species are currently identified in the taxonomy of bacteria and archaea using the polyphasic approach, which considers an organism's various dimensions, including its phenotypic, genotypic, and chemotaxonomic features (Tindall et al., 2010). Since genetic data reveals the evolutionary links between various lineages, genotypic characterization is a crucial part of characterizing species in this process (Chun & Rainey, 2014). Genotypic characterization has been greatly influenced by emerging sequencing technologies. New sequencing methods have allowed for faster and more efficient genotyping of the entire organism’s genome and open opportunities to improve many aspects of microbiology research (Scheben et al., 2017).

The sequencing of DNA and proteins began in the 1970s with the sequencing of the virus Lambda by Sanger et al. (1977). Around this time, DNA sequencing was done for organisms with tiny genomes, such as viruses, but it was not possible to sequence a bacterium completely due to economic and technological constraints. Later, however, advances in sequencing technologies and the deciphering of the human genome made it possible to sequence the entire bacterial genome. These techniques, termed next-generation sequencing technologies, have a high throughput, can produce more sequences, and are also less expensive. The technologies used in next-generation sequencing techniques, including single-molecule DNA sequencing and sequencing by ligation, apply a sequencing-by-synthesis approach. This technique produces clonal DNA colonies by attaching DNA molecules and primers on a slide and amplifying them with DNA polymerase. Several different kinds of fluorescently tagged reversible-terminator nucleotides are introduced, and the incorporated nucleotides are photographed in order to assess the DNA sequence. The subsequent cycle can begin after the fluorescent dye with the terminal 3' blocker is chemically removed from the DNA. A completely contiguous DNA sequence of the microbe's chromosome is what a genome sequencing study aims to achieve. After a microbial genome has been mapped, the next step is to annotate it; genes or proteins can be used to infer structural, physiological, and other biological information. Predicted protein-coding sequences, also known as open reading frames, are identified in the first step of the annotation process using bioinformatics analysis and tools like BLAST (Basic Local Alignment Search Tool). Because these species lack introns and have a high gene density, it is much simpler and more precise to identify open reading frames in the genomes of bacteria and other prokaryotic organisms. Only a portion of the open reading frames in the genome's sequence actually code for proteins, and the next step in the annotation process is to predict their activities by comparing them to genes with corresponding known functions in databases (Donkor, 2013).

Genome sequences have significantly improved the categorization of species compared to earlier methods because they clarify operational features of taxonomic groups and effectively solve inconsistencies in the phylogeny of upper taxa (Whitman, 2015). The study of the epidemiology, pathophysiology, and microbiological characteristics of bacteria is built on the precise species and variant assignment of bacterial isolates, enabling improved worldwide monitoring and comparative genomics. For example, Wu et al. (2020) updated the complicated taxonomy of *Enterobacter* species and subspecies based on whole-genome taxonomic analysis. Utilizing the genome data comes once a genome has been sequenced and annotated; it provides the fundamental knowledge necessary to comprehend the biology of the organism (Donkor, 2013). The entire genome of each microorganism (metagenome) in an environmental sample is used by the most recent genome sequencing technology in microbiology. New species can be identified, and genes essential for the community's survival can be characterized by sequencing every gene in a microbial population. The way we research bacteria has been changed by genome sequencing technologies (Antoniewicz, 2020). Similar to how information technology gave rise to Google Maps, which allows us to

find out specifics about locations across the world, genetic information from genome sequencing is utilized to construct maps of various organisms' genomes. Studies are no longer restricted to the small percentage of bacteria that will grow in a laboratory because they can be carried out on a lone microbe that has been directly derived from its natural home.

From a microbiology perspective, genome sequencing still needs a significant amount of DNA template, which is acquired from uniform cell cultures. The majority of these species, however, could not be sequenced because it is predicted that the majority of all bacterial species remain uncultivated due to unknown growth requirements. The advent of 16S ribosomal RNA gene PCR analysis, which allowed for the amplification and sequencing of an extremely informative gene from the majority of bacterial species, transformed the study of microbial genomics. Although phylogenetic trees could be built using the 16S rRNA gene sequence, investigations were restricted to this one gene. Metagenomics, a technique that involves sequencing all the DNA from an environmental sample, made it possible to make yet another significant advancement. The benefit of metagenomics is that it offers sequences for all of the genes present in environmental populations. However, the synthesis of genes and individual genomes continues to be difficult because of the tremendous diversity of several microbial communities. Short reads from next-generation sequencing are mostly used in metagenomics. The method becomes constrained in its capacity to precisely identify differences between bacterial strains, such as the existence of novel genes, as the richness of the microbial community rises.

## Roles of Molecular Systematics in Microbiological Research

Molecular systematics has become the primary way to determine evolutionary relationships among microorganisms because morphological and other phenotypic characters are either absent or change too rapidly to be useful for phylogenetic inferences (Eisen, 1995). In systematic molecular studies, proteins and DNA are the two molecules that have been most studied. These molecules contain phylogenetic information in a linear array. These arrays are informative because they reflect indirectly (in the case of proteins) or directly (in the case of DNA) the heritable developmental program of the organisms (Powell, 1991). Moreover, systematic molecular studies and the molecule of choice for most such studies of microorganisms have been the small subunit of the rRNA (SS-rRNA), which revolutionized the understanding of the diversity and phylogenetic relationship, particularly to microorganisms (Eisen, 1995). The progress in molecular biological tools such as DNA hybridization, polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP), randomly amplified polymorphic DNA (RAPD), allozyme data, microsatellite DNA provide various molecular data to gather phylogenetic relationships among taxa (Hwang & Kim, 1999).

The study of Nuñez et. al., 2017 used 16S rRNA gene-based ribotyping, oligotyping, and multilocus sequencing analysis (MLSA) to determine phylogenetic relationships and phylogenetic species boundaries of *Acidithiobacillus* spp. These sulfur-oxidizing acidophilic bacteria thrive in natural environments of low pH, and their properties have long been harnessed for the biotechnological processing of minerals. In another case, the

characterization of members of the genus *Bifidobacterium*, sequencing of the 16S rRNA gene, and some other genes considered to be molecular markers was investigated (Sidarenka et al., 2008). In addition, among the tested procedures, rep-PCR proved to be the most effective and reliable, allowing rapid differentiation of *Bifidobacterium* strains (Jarocki et al., 2016). Furthermore, rep-PCR fingerprinting using the BOXA1R primer can be considered a promising genotypic tool for the identification of a wide range of *Bifidobacteria* at the species, subspecies, and potentially up to the strain level (Masco et al., 2003). The enormous demand for probiotic bacteria and the accurate identification of these specific bacteria to produce high-quality products containing living microorganisms requires rapid and accurate identification of specific bacteria.

In the study of Carvajal & Galvez (2015), which characterized *Methylobacterium*, the use of the 16S rRNA gene sequence was preferred since it provides more accurate, elucidated identification and definition of the difficult bacterial isolates. The study revealed that through the identification of strains of *Methylobacterium* which are mostly found in the leaf surfaces of plants and have been shown to produce phytohormones (i.e., cytokinin and auxins). Furthermore, the identification of *Methylobacterium* strains showed that some strains could metabolize long chains of aliphatic hydrocarbons and thrive in polluted environments, therefore its potential as pollution indicators. Hwang et al. (2022) studied three species of diatoms from Mountain Streams in South Korea using both morphological (LM and SEM) and molecular approaches focused on small subunit (SSU) rRNA and ribulose bisphosphate carboxylase (rbcL) genes. The results obtained from the molecular analysis of this study showed that the three species had clear differences in phylogenetic distance. On the other hand, in algae taxonomy, the use of SSU nrDNA in establishing relationships between members of Gracilariceae was first carried out at the family-to-species level in red algae (Maggs et al., 2007).

## Roles of Molecular Systematics in Microbiology in Public Health

The study by Allard and Brown (2020) stated that the field of epidemiology, public health, and food safety needs more interdisciplinary teams with expertise in molecular systematics. The ten public health articles included in this study revealed that MLST, secondary to whole genome sequencing, is the most utilized molecular technique for health surveillance and outbreak investigation.

The studies of Chilam et al. (2021) discussed the genomic characterization of the two medically important nosocomial pathogens, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Whole genome sequencing was performed to determine all genes present in the isolate, including antimicrobial resistance (AMR) genes and virulence genes. Integrons were also detected, which carry the series of AMR genes, which can either be chromosomal in nature or transmitted via plasmids. Through MLST analysis, the prevailing clonal systems and sequence types of these two pathogens in the country were assessed. In addition, the results of whole genome sequencing results permit further investigation of the makeup of the organism through bioinformatics analysis. Furthermore, the phylogenetic relationship among

isolates shows strong evidence of *P. aeruginosa* and *A. baumannii* outbreak occurrence in multiple hospital sites in the country. The results showed that the isolates were from a common source since the single nucleotide polymorphism (SNP) difference is very minimal, which ranges from 0-1.

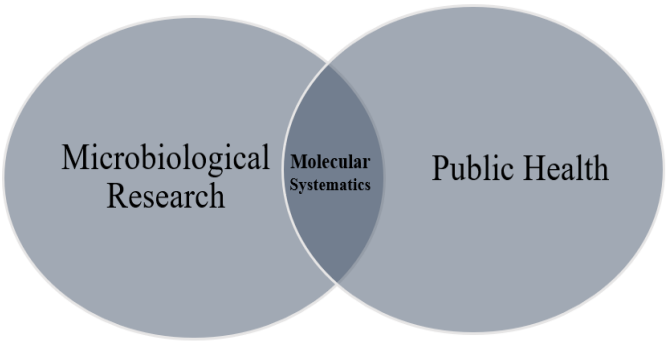
Jamoralin et al. (2021) sequenced the whole genomes of 21 *N. gonorrh*ea isolates collected in 2013–2014 by ARSP. The multilocus sequence type, multiantigen sequence type, presence of determinants of antimicrobial resistance, and relatedness among the isolates were all derived from the sequence data. Their study led to the placement of Philippine genomes within the global lineage A and the determination of an international transmission route.

## Moving Forward with Molecular Systematics in Microbiology in the Philippines

The Department of Health- Research Institute for Tropical Medicine (RITM) and the Department of Science and Technology – Industrial Technology Development Institute were two of the leading government institutions that facilitate public health and biological research development. The Antimicrobial Resistance Surveillance Reference Laboratory (ARSRL) is one of the national reference labs situated at RITM and conducts real-time surveillance of medically important pathogens. ARSRL is partnered with a sentinel laboratory-based antimicrobial resistance surveillance on aerobic bacteria from clinical specimens. Currently participating in the program are 24 sentinel sites and two gonococcal surveillance sites, representing 16 out of the 17 regions of the country. This laboratory utilizes SaTScan software which performs prospective real-time or time-periodic disease surveillance for the early detection of disease outbreaks. The detected cluster of isolates was sent out to ARSRL and was then subjected to whole-genome sequencing. Sequence data were used for phylogenetic analysis and computation of SNPs differences. The results of the bioinformatics analyses were communicated as soon as possible to the infection control team of the hospital for proper management.

On the other hand, the Industrial Fermentation Technology Section (IFTS), a unit under the Environment and Biotechnology Division (EBD) of the DOST-ITDI mandated to conduct R&D and technical services on biotechnology. One of the main research conducted in this institute is the identification and phylogenetic analysis of actinomycetes from various sources. In addition, EBD also dwells into cataloging possible Philippine strains of Zika and African swine fever viruses and coconut cadang-cadang viroid through genome sequencing. These developments and programs showcase the utilization and importance of molecular systematics to push forward research and surveillance in the country.

**SYNTHESIS**




The figure above shows that the field of microbiological research and public health both utilizes the techniques of molecular systematics. In congruence with the collected articles in this review, rapid and accurate identification of microorganisms was the primary goal of microbiological research for the utilization of these methods. While it can be noted that, for public health purposes, molecular systematics involves the initial step of identifying the isolates followed by the assessment of the relationship among the group of organisms. Then the generated phylogenetic tree and SNPs matrix would reveal the occurrence of the outbreak in a specific spatiotemporal event.

## Conclusion

Molecular systematics and its methodologies unleash the full capacity of investigation in the microbiological field and public health setting. The main utilization of molecular systematics in these two areas of study involves identification, assessment of phylogenetic relationships, and outbreak investigation among microbial isolates.

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