2 DNA–DNA Reassociation Methods Applied to Microbial Taxonomy and Their Critical Evaluation

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2.1 Introduction

DNA–DNA reassociation techniques are used for many purposes, but in the field of microbial systematics they are in most cases linked to the circumscription of prokaryotic species. Actually, as we will see, the use of whole genome hybridizations in the definition of prokaryotic species has had an enormous influence since the origin of the polythetic classification system (Rosselló-Mora and Kämpfer 2004). The importance of morphology in the middle of the eighteenth century was substituted for that of biochemical properties at the beginning of the nineteenth century; and subsequently the emerging "modern spectrum" techniques emphasized the importance of genetic measurements, such as DNA–DNA reassociation experiments. However, after almost 50 years of the application of these techniques to circumscribe species, there is increasing reluctance to use them because of the intrinsic pitfalls in the methods (e.g. Stackebrandt 2003; Stackebrandt et al. 2002). Consequently, the question that arises is: if DNA reassociation techniques are to be substituted, what will take their place? However, in my opinion, it is still too soon to substitute these techniques because of several reasons: (a) the use of such parameters in the definition of species has been of paramount influence and has actually determined the size and shape of what we call 'species', (b) there are almost 5,000 species described (Garrity et al. 2004), many of them based on reassociation experiments, and the legitimacy of new circumscription methods should be validated and (c) the alternatives proposed are not yet standardized and tested sufficiently enough to offer a reliable, pragmatic and easy to use circumscription tool. Any new technique with the potential to act as a substitute for DNA–DNA reassociation experiments should demonstrate that: (a) it is more reliable, workable and pragmatic, (b) it does not radically change the present classification system and (c) it leads to results that fit into a genomically based perspective without losing sight of the organisms themselves. Any

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intended substitution of a technique that has implications for the circumscription parameters that have served as a basis for the establishment of the current taxonomic system should also take into account the purpose of taxonomy. The end result itself is to provide a system that is operative and predictive; and the information behind a name should be more than a mere set of genes with no meaning. What has hitherto been constructed is a classification system based on the circumscription of taxa when the overall information collected indicated that such circumscription would be enough to recognize them as unique and identifiable. Behind a species name there is more than a binomial, there is a collection of data that allows identification from several independent sources that gives a prediction of how an organism may be and might behave. Our system is perhaps not perfect and deserves improvement, but as already noted "it is the envy of those who wish to implement similar systems in botany or zoology" (Euzéby and Tindall 2004).

DNA–DNA reassociation techniques, also known as DNA–DNA hybridization techniques, are based on an attempt to make raw comparisons of whole genomes between different organisms in order to calculate their overall genetic similarities. Just after the discovery of the intrinsic properties of DNA (i. e. information content and secondary structure resilience), a good number of techniques were developed and applied to microbial taxonomy in order to circumscribe its basic unit, the species. At that time, it was believed that such genetic comparisons would render more stable classifications than those simply based on phenotypic similarities (Krieg 1988). There is no doubt that the first attempt to elucidate taxonomic relationships based on single-stranded DNA reassociation conducted by Schildkraut et al. (1961) was a breakthrough for microbial systematics and for the construction of the current microbial classification system. They demonstrated that duplex formation between the denatured DNA of one organism and that of another organism would only occur if the overall DNA base compositions were similar and if the organisms from which the DNA was extracted were genetically related. At the time when a monothetic classification was abandoned in favour of a polythetic (or phenetic; Rosselló-Mora and Amann 2001) classification, these developments in DNA techniques led to microbial taxonomists extending the definition of the species by using reassociation results and by determining the GC mole percentage of each individual genome. The great practical advantage seen in DNA–DNA hybridization experiments was that the results did not show the continua often observed between groups defined by phenotypic characteristics, but instead the genomes appeared clustered in discrete groups, whether organisms tended to be closely related or not (Krieg 1988). Since then, such techniques have routinely been applied in most of the new species characterizations, especially those that involved new taxa in already existing

genera and/or those where more than a single isolate was used to circumscribe the taxon. The application of these techniques to circumscribe species was reinforced by a recommendation from an ad hoc committee on systematics (Wayne et al. 1987). In fact, the committee (using ΔT_m to indicate melting temperature increment) stated that "the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less ΔT_m. Both values must be considered. Phenotypic characteristics should agree with this definition and would be allowed to override the phylogenetic concept of species only in a few exceptional cases". In addition, they reinforced that "it is recommended that a distinct genospecies that cannot be differentiated from another genospecies on the basis of any known phenotypic property not be named until they can be differentiated by some phenotypic property". That recommendation had two main effects. On the one hand, it forced descriptions based on both genomic and phenotypic properties but, on the other hand, it unwittingly created the belief that a rigid boundary of 70% genome similarity would be sufficient for the recognition of species. Both aspects have had an enormous influence on prokaryotic taxonomy.

Emerging techniques at the end of the twentieth century, such as rRNA gene sequencing and phylogenetic reconstructions, were expected to help in the replacement of DNA-DNA reassociation experiments. However, it was soon realized that, due to the length and information of the molecule, the resolution power needed to discriminate different species within a genus was not always adequate (e.g. Amann et al. 1992; Fox et al. 1992; Martínez-Murcia et al. 1992). For these reasons, it was accepted at that time that no other methodology could replace genome similarity analysis (Stackebrandt and Goebel 1994). It has always been clear that the best way to understand similarities would be to truly compare whole genome sequences (e.g. Owen and Pitcher 1985), a fact that has nowadays almost become possible. The increasing number of completely sequenced genomes allows such comparisons and the first speculations on how species can be circumscribed by this newly emerging information (Konstantinidis and Tiedje 2005; Santos and Ochman 2004; Stackebrandt et al. 2002; Zeigler 2003). However, all these new circumscription attempts should be previously validated by contrasting them with the criteria used to construct the current taxonomic schema.

DNA-DNA reassociation experiments have often been criticized due to their high experimental error and their failure at generating cumulative databases (e.g. Sneath 1989; Stackebrandt 2003). However, their use has never been abandoned because no other alternative has been either found or tested. In order to illustrate how often DNA-DNA reassociation experiments are still used to circumscribe species, a survey on all the publications that appeared in 'Int. J. Syst. Evol. Microbiol.' during 2004 has been under-

Table 2.1. 'Int. J. Syst. Evol. Microbiol.' survey: absolute numbers and percentages of articles or new descriptions that were published in the six issues of vol 54 of the journal during 2004

Articles with new descriptions	305	
Articles with reassociation experiments	199	65%[a]
Articles without reassociation experiments	106	35%[a]
Spectrophotometric reassociation experiments	67	34%[b]
Non-radioactive microtitre-plate hybridizations	96	48%[b]
Non-radioactive filter methods (chemiluminescence)	9	5%[b]
Radioactive filter, S1, or hydroxyapatite methods	27	14%[b]
New species	351	
New species with a single isolate	191	54%[c]
New genera	65	
New 'candidatus'	17	

[a] percentages refer to the 305 articles with new descriptions

[b] percentages refer to the 199 articles where reassociation experiments were performed

[c] percentages refer to the total number of 351 new species classifications

taken (Table 2.1). In that year, around 305 articles appeared that compiled the description of about 351 new species, 65 new genera, and 17 new 'candidatus'. Among all these new species descriptions, about 65% of them used DNA–DNA reassociation experiments. From the 35% of the remaining descriptions where no reassociation was used, more than 75% were based on a single isolate and more than half corresponded to new genera. In such cases, the rationale for taxa descriptions were mainly based on 16S rDNA sequence dissimilarities. However, it is also worth noting that among all the descriptions where DNA–DNA reassociation was used, nearly 60% of them were also based on a single isolate. In these cases, the use of hybridizations was to show enough dissimilarity to their closest relative species.

There is a desire to replace DNA–DNA reassociation for other more accurate techniques (Stackebrandt et al. 2002) but its use still cannot be avoided. Consequently, this is a timely review concerning existing techniques, their pitfalls and the meaning of their results. In addition, the possibility to replace them will also be discussed.

2.2 Semantic Considerations

Prokaryotic taxonomy, like eukaryotic taxonomy, is filled with semantic misuses. There are several examples that in some respect are responsible for the so-called 'species problem': (a) the use of homology as a synonym of similarity, (b) the persistent homonymy of the term species and (c) the

synonymy between concept and definition. Although these issues will be thoroughly discussed elsewhere, it is worth providing some clarifications at this point:

1. Homology vs similarity: since the early days of the interpretation of DNA–DNA reassociation results, homology and similarity have been used as synonyms. However, it was soon noted that the use of the term homology would not be appropriate for interpreting hybridization results, because there was no certainty that bound stretches of DNA from different organisms would contain identical nucleotide sequences and the use of terms such as relatedness or DNA binding would be more accurate (Brenner and Cowie 1968; De Ley et al. 1970). However, these recommendations were not taken into account and for decades the term homology has been used to express DNA–DNA reassociation results. Later, there was again the temptation to abandon the term homology (Stackebrandt and Liesack 1993) by arguing that the values observed were not linearly correlated with sequence identity. Homology is not a measurable parameter: either two characters (in this case sequences or DNA fragments) are homologous or not, which means that either they have the same evolutionary origin or not (Fitch 2000; Mindell and Meyer 2001; Tindall 2002). Homology basically has an evolutionary meaning and thus cannot be applied either as a synonym for sequence identity or to express DNA–DNA reassociation results. The term similarity is perhaps the best choice because it does not imply any evolutionary nor phylogenetic meaning. Despite the reiterated recommendations, there are still quite a few publications that wrongly use the term homology.

2. Homonymy of the term species: perhaps the most important cause of the 'species problem' is the persistent homonymy (Reydon 2004). This means that different scientific disciplines adopt different concepts to embrace their devised units, but the same term 'species' is given to all of them. This has always been regarded as a clear case of pluralism (Brigandt 2002; Ereshefsky 1998; Mishler and Donoghue 1982; Reydon 2004). For some, it would be better to eliminate the term species and each scientific discipline should instead adopt a unique and specially tailored basic unit, such as 'biospecies', 'ecospecies' or 'phylospecies' (Ereshefsky 1998). However, for others, pluralism is still an adequate choice, with the term 'species' being kept for general-purpose classification, which should retain binomials as a property of the taxonomic system (Brigandt 2002). These problems, which have been thoroughly discussed in eukaryotic taxonomies, are well represented when classifying prokaryotes. Actually, what taxonomists mean by a species does not satisfy, for instance, microbial ecologists or population geneticists, although it would probably not be possible for these groups to come to any mutual agreement on terminology. It is also important to note that, for example "evolution was inferred from the classification, not vice

versa" (Sneath 1988) and thus the ultimate concept of 'species' is a property of taxonomy. These disagreements are the basis for most of the discussions on the adequacy of the current species concept in use (Rosselló-Mora and Kämpfer 2004) and, therefore, most probably it would be recommendable to adopt a clear pluralistic approach. Taking into account that the term and idea of 'species' is the basal taxonomic unit originally devised to support a universal hierarchic system (Ereshefsky 1994), the main arguments expressed here are within the framework of taxonomy and refer to the species concept currently applied to the classification of prokaryotes. Perhaps the most updated version of the prokaryotic species concept is "a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions" (Stackebrandt et al. 2002). The whole critical viewpoint here revolves around the adequacy of DNA–DNA reassociation experiments to circumscribe genomically coherent groups.

3. Concept and definition: another exponent example of semantic misunderstanding is the confusion between concept and definition. Both terms are often used as synonyms, but it is important to take into account that distinguishing them may very much help in clarifying our prokaryotic species 'problem'. The species concept is the idea that explains and circumscribes the patterns of recurrence observed in nature. It is the essence of what we think is the basic unit for constructing an operative and predictive classification. Within the concept, we should find the reasons for including or excluding naturally occurring individuals within a category. However, the species definition is the way we recognize that individuals belong to a category. The definition provides a set of parameters that are sufficient to recognize that a certain group of individuals belong to a recurrent pattern in nature. Actually, this responds in the most pragmatic way to identify what we think is a unit. Our reductionistic approach to understanding nature allows us to formulate the simplest way to recognize units (Rosselló-Mora 2003). For example, in this chapter, 'genomic coherency' applies to the concept, whereas the relaxed (or not) results or values of DNA–DNA reassociation experiments would apply to the definition. For example, changing the method and parameters to recognize coherent genomic groups, such as substituting DNA–DNA reassociation experiments (e. g. MLST), would result in a change in how we define species but not how we conceive them. The concept remains the same.

2.3 DNA–DNA Reassociation Measurement, Parameters and Methods

During the almost 50 years of use of whole genome hybridization studies for microbial taxonomy, quite a few techniques have been developed (Table 2.2). All such techniques have in common the measurement of the extent and/or stability of the hybrid double-stranded DNA resulting from a denatured mixture of DNAs incubated under stringent conditions that allow only renaturation of complementary sequences. Actually, the use of different techniques, and their comparisons have been extensively discussed (e.g. Brenner 1978; De Ley and Tijtgat 1970; Goris et al. 1998; Grimont 1988; Grimont et al. 1980; Johnson 1985, 1991; Owen and Pitcher 1985; Stackebrandt and Liesack 1993; Tjernberg et al. 1989). As will be clarified later and despite any apparent diversity, all methods rely on a few common properties with the differences between them being basically variations in the DNA labelling type and/or the measurement technique. It seems that with time, the multiple techniques published have been developed following the need to simplify the manipulation procedures, and allow a larger number of simultaneous measurements.

There are two main strategies for performing reassociation experiments: those where the hybridization reaction is carried out in free solution and those that imply previous fixation of the test DNA onto a solid surface. Among the free-solution methods, the most ancestral required one of the test DNAs to be labelled with heavy isotopes; and the separation of homologous renatured strands from the hybrids was carried out under buoyant density ultracentrifugation procedures (Schildkraut et al. 1961). However, better accuracy in the measurement of hybrid molecules was achieved by the use of radiolabels. A labelled DNA, commonly sheared into small single-stranded polynucleotide molecules, is hybridized against an excess of unlabelled high-molecular-weight target DNA. Double-stranded DNA is then separated from single-stranded unhybridized DNA either by the use of a selective binding to hydroxyapatite (Brenner et al. 1969b), or by the selective digestion of single-stranded DNA with nuclease S1 (Crosa et al. 1973; Popoff and Coynault 1980). Both strategies gauge the measurement of the extent of labelled DNA that has hybridized against an unlabelled target and its comparisons against homologous reassociations. Due to methodological and health concerns, the use of radiolabels is not easily implemented in laboratories, promoting the development and establishment of non-radioactive methods. For example, there is a non-radioactive and miniaturized method equivalent to the original hydroxyapatite method where DNA is double-labelled with biotin- and digoxigenin-modified nu-

Table 2.2. Comparison of methods used for DNA–DNA reassociation experiments, description of their methodological basis and their advantages and disadvantages. *M* Method, *L* labelling, *Ms* measured parameter

Method	Short description	Remarks
Free-solution methods		
M: buoyant density L: heavy isotopes Ms: RBR Schildkraut et al. (1961)	One of the two genomes to test is labelled with a heavy isotope. Renatured strands are separated on a caesium chloride gradient by ultracentrifugation. Quantification is made on the relative amounts of mixed DNA of intermediate molecular weight.	The method was rapidly abandoned due to the advantages of the newly developed methods.
M: hydroxyapatite L: radioactive isotope Ms: RBR; ΔT_m Brenner et al. (1969b) Lind and Ursing (1986)	Radioactively labelled DNA is hybridized in solution under stringent conditions against unlabelled DNA. Single DNA strands are separated from renatured double DNA strands on the basis of physical affinity of a hydroxyapatite matrix. Quantifications are made upon the relative amounts of hybrid DNAs formed in respect to the homoduplex renatured DNA. Likewise, temperature denaturation profiles can be performed to quantify the thermal stability of the hybrids in respect to the homoduplex (ΔT_m). Reference DNAs have to be labelled with radioactive isotopes by either nick translation or iodination. Quantifications are done as specific radioactivity (cpm).	Advantages: multiple simultaneous hybridizations can be performed. The method allows a short protocol for RBR measurements and a longer one for ΔT_m measurements. Hybridizations can be done under stringent conditions and without adding extra components (e.g. formamide, blocking reagents, etc.). The hybridization temperature is not restricted and can be as high as needed. Disadvantages: reference DNAs should be labelled with radioactive isotopes.

Table 2.2. (continued)

Method	Short description	Remarks
M: hydroxyapatite/microtitre plate L: digoxigenin–biotin Ms: RBR Ziemke et al. (1998)	The method is basically a modification of the Brenner et al. (1969b). Double-labelled DNA (biotin/digoxigenin) is hybridized against unlabelled DNA. Single- and double-strand DNAs are separated by the use of hydroxyapatite. Quantifications of both fractions are done on streptavidin-coated microtitre plates, using an alkaline phosphatase-based colorimetric enzymatic bioassay. Reference DNAs have to be double-labelled by using nick translation and modified nucleotides bound to digoxigenin or biotin. Quantifications are done by reading the absorbance at 405 nm in a microtitre plate reader.	Advantages: the same as the radioactive method, but no ΔT_m can be performed due to the lower sensitivity of the method. Labelling is performed with non-radioactive labels. Disadvantages: double-labelling should be done with nick-translation and only RBR can be determined.
M: spectrophotometry L: none Ms: RBR; ΔT_m De Ley et al. (1970) Huß et al. (1983)	Renaturation rates of equimolar mixtures of two DNAs are measured by the resulting slope recorded by the decrease in absorbance at 260 nm, and compared to those of the respective homoduplexes.	Advantages: there is no labelling step in the method and DNAs can be used almost without any manipulation. Disadvantages: Special spectrophotometers are needed. The amounts and quality (length and purity) of the two DNAs in the hybridization should be identical, only pairwise results can be achieved. Perhaps the most time-consuming and DNA quantity- and quality-demanding technique.

Table 2.2. (continued)

Method	Short description	Remarks
M: Fluorimetric L: none Ms: ΔT_m González and Sáiz-Jiménez (2004)	Method comparable to that of spectrophotometry. Unlabelled DNAs are hybridized under stringent conditions. Hybrid denaturation rates are measured by following the decrease of fluorescence of double-strand DNA specific fluorescent dye (e. g. SYBR green I). Quantification is made upon the difference between the thermal mid point of the homoduplex and that of the hybrids (ΔT_m).	Advantages: relatively low amounts of DNAs are needed, there is no labelling step and multiple simultaneous experiments can be performed. Disadvantages: The technique needs the use of real-time fluorescent detectors, such as real-time PCR thermocyclers. As for the spectrophotometric method, DNAs should have identical quality and quantity conditions. The method is new and needs to be validated before being compared with those in routine use.
M: endonuclease L: radioactive isotopes Ms: RBR Crosa et al. (1973): S1-TCA Popoff and Coynault (1980): S1-DE81	Labelled and unlabelled DNAs are hybridized in solution under stringent conditions and further digested with a single-strand-specific endonuclease. Double-strand DNAs are then separated from free radioactive nucleotides either by precipitation (S1-TCA) or filtration (S1-DE81). Amounts of hybrid DNAs are referred to renatured homoduplex DNA. Labelling and quantification are done as in the hydroxylapatite method.	Advantages: the same as for the hydroxyapatite method. Disadvantages: reference DNAs should be labelled with radioactive isotopes.
Bound-DNA methods		
M: agar embedded L: radioactive isotopes Ms: RBR Bolton and McCarthy (1962) Brenner et al. (1969a)	Denatured DNA is embedded in agar, left to solidify and then disaggregated into small fragments by pressing through a mesh. The small fragments of agar containing unlabelled DNA are hybridized against radioactively labelled DNA in solution. Once hybridization is done, the agar is washed and the radioactivity quantified.	Advantages: accuracy of the radioactivity measurements. Disadvantages: accessibility of labelled DNA to the embedded DNA, difficulties in incubating the samples at high temperatures.

Table 2.2. (continued)

Method	Short description	Remarks
M: membrane filters L: radioactive isotopes Ms: RBR; ΔT_m Johnson (1981) Owen and Pitcher (1985) Tjernberg et al. (1989)	Unlabelled and denatured DNAs are covalently bound to a membrane filter and then hybridized against free radioactively labelled DNA. Hybridization is done under stringent conditions where, for example, competition with unlabelled DNA can be performed. Hybridization solution includes blocking reagents to avoid unspecific binding to the membrane filter. RBR measurements are done in comparison to the homoduplex reaction. Likewise, temperature denaturation profiles can be performed to quantify the thermal stability of the hybrids with respect to the homoduplex (ΔT_m). Labelling and quantification are done as in the hydroxyapatite method. Additionally, sample-specific activity can also be measured by the use of autoradiography accompanied by densitometric analysis.	Advantages: multiple simultaneous hybridizations with a single-labelled DNA can be performed. These methods calculating thermal stability of the hybrids are independent of the quality of the bound DNA [e.g. Tjernberg et al. (1989) use lysed cell cultures instead of purified DNA]. Disadvantages: the use of radiolabels and, for RBR calculations, the amount of bound DNA may not be equivalent for the different samples and may saturate the dot. Additionally, DNA can be released from the membrane during the incubation/washing steps, therefore biasing the results.
M: membrane filters L: non-radioactive labels Ms: RBR Jahnke (1994) Cardinali et al. (2000) Gade et al. (2004)	Hybridization conditions are similar to those of the radioactive method, only the detection steps are a bit more tedious. Quantifications are done by using colorimetric enzymatic bioassays and differences are mainly due to the type of detection. There are several quantification possibilities, e.g. soluble enzymatic product (Jahnke 1994), chemiluminescence, film development and densitometric analysis (Cardinali et al. 2000), insoluble precipitated enzymatic product and densitometric analysis (Gade et al. 2004), among others.	Advantages: the same as the radioactive methods, but using non-radioactive labels instead. Disadvantages: the same as the radioactive methods, but with more incubation/washing steps. Due to the sensitivity of the detection only RBR can be determined. Densitometric measurements might not be accurate enough. Being an enzymatic reaction, the development should be done during the period of time when the reaction is linear.

Table 2.2. (continued)

Method	Short description	Remarks
M: microtitre plate-bound DNA L: photobiotin Ms: RBR Ezaki et al. (1989) Adnan et al. (1993) Kaznowski (1995) Christensen et al. (2000)	Unlabelled and denatured DNAs are adsorbed or covalently bound to the wells of a microtitre plate where the hybridization will take place. Reference DNAs are labelled with photobiotin and hybridized against bound DNA. To reduce hybridization temperatures and in order to maintain stringency, formamide is used. The amount of hybridized DNA is revealed after a fluorometric or colorimetric enzymatic bioassay.	Advantages: depending on the protocol, hybridizations are reduced to a few hours. Multiple simultaneous measurements can be performed. Labelling is performed with non-radioactive labels. Disadvantages: as in any assay where DNA is bound, and due to the large number of steps and incubation times, DNA can be released therefore biasing the results. Only RBR can be calculated while no temperature profiles can be used. Hybridization has to include the use of denaturing agents such as formamide to avoid incubations at high temperatures.
M: microtitre plate-bound DNA L: digoxigenin Ms: ΔT_m Mehlen et al. (2004)	Reference and test DNAs are digested with *Sau*3A restriction enzyme and then ligated to oligonucleotide linkers that serve as a target for amplification purposes. Genomic DNAs are then amplified, and the reference DNA is labelled with digoxigenin modified dUTP. Non-labelled DNAs are covalently bound to microtiter plates and hybridized with free-labelled reference DNA. Hybridization is followed by different washes with buffer of ion concentrations of increasing stringency. Detection of bound DNA is revealed after a colorimetric enzymatic bioassay.	Advantages: in principle, there is no need for high quantities of genomic DNAs. Labelling is performed with non-radioactive labels, and detection can be simply done with a standard microtitre reader. The calculations of melting temperatures minimize the effects of unspecific binding, and are unaffected by different amounts of unlabelled microtitre-bound genomic DNA. Disadvantages: any problem derived from the amplification procedure. No RBR can be calculated. Hybridization has to include the use of denaturing agents such as formamide to avoid incubations at high temperatures.

cleotides; and the detection is simply undertaken as a bioassay in microtitre plates (Ziemke et al. 1998). As an alternative to labelling DNA, a spectrophotometric method was developed by De Ley et al. (1970) where a mixture of two unlabelled DNAs of identical quality and concentration are denatured, and their renaturation is optically followed under stringent conditions with a special spectrophotometer. The measurement of reassociation is made by the decrease in absorbance that single-stranded DNA shows when it renatures as a double strand. The extent of hybrid molecules is extrapolated from the comparisons of the differences in the reassociation rates of homologous and heterologous DNAs. Recently, a new fluorometric method that uses a real-time PCR thermocycler has been developed with a similar basis as the spectrophotometric method (González and Sáiz-Jiménez 2004). This method is based on measuring the thermal stability of the hybrid molecules with the use of SYBR green I. Although this method is still to be validated by evaluating the results with other techniques, preliminary comparisons indicate its adequacy (Jurado et al. 2005).

All methods implying fixed DNA rely on the same principle, where the denatured target DNA is bound to a solid surface and then hybridized against a labelled reference DNA in free solution. Labelled DNA is dissolved in a solution with an ionic strength that provides enough stringency to allow only renaturation of complementary strands at a given temperature. Additionally, the hybridization buffer includes several coating compounds that hamper unspecific binding of labelled DNA to the DNA-free solid surface. The first experiments were performed with agar as the solid surface for binding DNA (Bolton and McCarthy 1962). However, such a supporting matrix was rapidly abandoned in favour of the use of macroporous supports such as nitrocellulose or Nylon filters which provided covalent surface binding of the DNA, and thus a minimization of the loss of the target DNA from the support. There are quite a few published procedures using membrane filters, with the main differences between them being basically the type of label for the reference DNA and thus the quantification measurement procedures. DNA can be radiolabelled and the hybridization extent can be either quantified by scintillation (e.g. De Ley and Tijtgat 1970), or by the densitometric measurement of the spot generated through autoradiography (e.g. Amann et al. 1992). However, similar methods have been developed by the use of non-radioactive labels, such as digoxigenin- or biotin-modified nucleotides; and the measurement is carried out after densitometric quantification of the spots generated, for instance, from chemiluminescence on X-ray films (e.g. Cardinali et al. 2000), or directly onto the membrane with a precipitated product (e.g. Gade et al. 2004). A colorimetric measurement with the combined use of microtitre plates has even been used (Jahnke 1994). More modern attempts to combine genomics technology with classic species circumscription have been undertaken by

the use of micro- or macroarrays (Cho and Tiedje 2001; Ramisse et al. 2003; Watanabe et al. 2004). However, most probably if the classic technologies are considered difficult to implement and only a few laboratories use them (Cho and Tiedje 2001; Stackebrandt 2003), the use of genomics technology might be even more restricted.

Finally, one of the most currently applied methods that implies immobilization of DNA onto a solid surface is the one that uses microtitre plates instead of macroporous membranes. The success of these methods relies on the possibility of performing fast and radioactivity-free assays, all in the same container. There are several published methods, but the most known and used is that of Ezaki et al. (1989) which binds the target DNA in the wells of a microtitre plate and the test DNA is labelled with biotin. First, measurements were undertaken by the use of fluorogenic substrates, but later these were substituted by a chemiluminescent substrate and by covalent binding onto the microtitre plate surface (Adnan et al. 1993). However, similar methods have been developed that use colorimetric reactions for the detection (Kaznowski 1995) which, importantly, reduce the cost of the equipment used. Lately, more sophisticated and reliable methods have been developed which allow experimentation with fastidious organisms whose DNA is difficult to recover (Mehlen et al. 2004) and, in this case, genomic DNA is previously amplified before being bound to the microtitre well. Then, digoxygenin-labelled reference DNA is used to perform the hybridization and the stringency is accomplished by washing with decreasing ion strength buffers, which allows a determination of melting profiles for hybrid molecules. Detection is achieved colorimetrically.

Depending on the method used, there are two main parameters that can be determined: the relative binding ratio (RBR) and the increment of melting temperature (ΔT_m). Sometimes the same procedure can provide both parameters, but most of the techniques just provide one or the other (Table 2.2). It is important to note that RBR values especially depend on the stringency of the method used. At a given ionic strength, hybridizations may be carried out under what are considered to be optimal conditions (25–30 °C below the melting point of the reference native DNA, i.e. T_m), under stringent or exacting conditions (10–15 °C below T_m), or under relaxed, non-exacting conditions (30–50 °C below T_m), although most results correspond to optimal-condition experiments (Schleifer and Stackebrandt 1983).

The RBR is the measurement of the extent of double-stranded hybrid DNA for a given pair of genomes relative to that measured for the reference DNA performed under identical renaturation conditions. RBR is expressed as a percentage, considering that the reference genome hybridizes 100% with itself. For those methods that use labelled DNA, large amounts of labelled DNA may still remain as single-stranded DNA after the hybridiza-

tion experiment; and then the binding ratio (BR) is calculated as the extent of double-stranded hybrid DNA in relation to the total labelled DNA added in each single experiment. RBR is then determined by comparing the percent reassociation of each heterologous reaction to that of the homologous reaction, which is considered to be 100%. Spectrophotometric methods calculate the extent of hybrid DNA by basically comparing the reassociation kinetics with those of homologous DNA. The RBR is the most used parameter in the circumscription of species.

A more reliable parameter to determine is the ΔT_m, simply because it is independent from the quantity and quality of the DNAs used for the experiment (Tjernberg et al. 1989). However, ΔT_m requires more time-consuming methods and is generally only achievable using radioactive labels. This parameter is a reflection of the thermal stability of the DNA duplexes. ΔT_m is actually the difference between the melting temperature of a given homologous DNA and that of a hybrid DNA. At a given ionic strength, the melting temperature of a DNA (or thermal denaturation midpoint, T_m; where 50% of DNA strands appear denatured) is directly related to its GC content (Schildkraut and Lifson 1965; Turner 1996). Hybrid DNAs tend to melt earlier. The less related a pair of DNAs, the higher the difference between their melting points (in degrees Celsius), in comparison with their corresponding homologues. This is because a lower base pairing will render a less thermally stable base complementation. When the measurements are carried out with a labelled reference DNA, the melting temperatures are solely related to the extent of base pairing and remain independent from the quality and quantity of each of the DNAs used for the hybridization. Consequently, the results of analysing melting profiles are very reproducible and less subject to experimental error than RBR. However, because of the technical difficulties, RBR is much more popular when trying to calculate raw genome similarities. In principle, the two parameters do not need to be related: RBR reflects the extent of double-stranded DNA with a base complementarity of less than 15% base mispairing (Stackebrandt and Goebel 1994; Ullmann and McCarthy 1973) and ΔT_m reflects the extent of sequence identity. However, it has been demonstrated empirically that there is indeed a linear correlation between them (e. g. Grimont 1988; Johnson 1989; Rosselló-Mora and Amann 2001; Tjernberg and Ursing 1989); and generally values of RBR above 50% correlate with a ΔT_m value below 4–5 °C.

To calculate ΔT_m, multiple-step washing profiles have to be carried out. However, a parameter named $\%DR_7$ was developed to simplify the washing profiles without losing accuracy in the measurements (Tjernberg et al. 1989). $\%DR_7$ is calculated after two steps of washing the hybridized molecules: the first wash is undertaken at 7 °C below the melting temperature of the reference DNA and a second wash is performed at 100 °C in order to achieve complete denaturation. $\%DR_7$ is the amount of DNA released in

the first step as a percentage of the total amount of eluted DNA. Thus, for a given pair of DNAs, the higher the $\%DR_7$, the less they are related. However, although this parameter could have been a good compromise between the accuracy of ΔT_m measurements and the simplicity of RBR calculations, it has never been applied to any great extent.

It is not easy to recommend a method, or a parameter, for circumscribing species when using DNA–DNA reassociation experiments. It is a question of the equipment that one possesses and the accuracy of the measurements that one wants to achieve. The sensitivity of radioactive measurements means these are the ones that provide the most accurate and reproducible data. Actually, such methods generally allow the measurement of both parameters, RBR and ΔT_m; and an additional advantage of using radioactive labels is that, when measuring melting temperatures, the results are independent of the quality and quantity of the DNA. It is even possible to use cell extracts directly and dot-blot them onto filters instead of previously having to isolate high-quality DNA (Rosselló et al. 1991; Tjernberg et al. 1989). The non-radioactive methods are currently the methods of choice, simply because of the security advantages of not using radiolabels. However, it has to be understood that the accuracy may be less because of the larger standard deviations of the experiments. Spectrophotometric methods, like real-time PCR measurements, require the determination of the exact amounts of the DNAs to be used; and for hybridization purposes both should have very similar conditions of quality. Additionally, they can only be undertaken as pair-wise assays, especially spectrophotometric methods; and for multiple determinations the experiment is quite time-consuming. Despite this, such experiments are currently some of the most popular for use in bacterial taxonomy (Table 2.1). The most used methods for determining genome similarities are those that imply attachment of the nucleic acids onto a solid surface, either on a filter or in microtitre plates (Table 2.1). All of them imply either adsorption or covalent attachment of the DNA onto a surface, with the expectation that: (a) identical test DNA amounts are attached per spot/well and (b) the loss of attached bound DNA due to washes and incubations is negligible. Despite this, these methods and especially those using microtitre plates (e.g. Christensen et al. 2000; Ezaki et al. 1989) are the most used (Table 2.1). Microtitre plate methods that use colorimetric bioassays, such as for instance modifications of the Ezaki method (Kaznowski 1995), or those that adapt radioactive methods to miniaturized non-radioactive procedures (Ziemke et al. 1998), may also be chosen because of the lower costs of the equipment used (i.e. regular microtitre plate readers are less expensive than special spectrophotometers, fluorometers or phosphor-imagers, among others).

Most of the methods have been thoroughly compared in order to validate their results (e.g. Christensen et al. 2000; De Ley and Tijtgat 1970; De Ley

et al. 1970; Ezaki et al. 1989; Goris et al. 1998; Grimont et al. 1980; Jahnke 1994; Mehlen et al. 2004; Tjernberg et al. 1989; Ziemke et al. 1998). From the comparisons, it can be deduced that the level of agreement is quite good, especially for those hybridizations of closely related strains; and generally values are above 50%. However, the level of agreement might decrease when the genome similarities are lower, just because the background of the techniques might be different. Additionally, it is important to take into account that the standard deviations are relatively high, especially for those techniques that are non-radioactive, and values might be as high as 8% (Christensen et al. 2000; Johnson 1991; Sneath 1989). Nevertheless, as will be argued later, the evaluation of the hybridization results may be better read as if evaluating, for instance, chemotaxonomic markers, where the patterns shown by the relative amounts of the components are of higher importance than those of each absolute value.

Finally, there is a belief that hybridization methods are difficult to implement in a regular laboratory because of the laborious procedures involved; and they are also of high cost because of the equipment required (e. g. Gillis et al. 2001; Stackebrandt 2003; Stackebrandt et al. 2002; Young 1998). However, I would argue here that this may be true only for such methods that require radiolabels, expensive spectrophotometers, fluorimeters, real-time thermocyclers, or X-ray film exposure and development. The methods adapted to colorimetric measurements (e. g. Kaznowski 1995; Mehlen et al. 2004; Ziemke et al. 1998), in contrast, require nothing more than the regular apparatus found in any microbiology laboratory, such as microtitre readers for visible light (which can be substituted by regular spectrophotometers), water baths, microfuges and even a low-cost thermocycler. The protocols developed are no more laborious than others dealing with molecular techniques; and, once DNA is isolated, the procedures can take one or at most two days.

2.4 Interpretation of Results and the Boundaries for Species Circumscription

The importance of the results generated by DNA–DNA hybridization techniques have been empirically emphasized after years of using such techniques. The original experiments were designed simply to understand raw genome similarities. However, soon the empirical observation that genomically coherent groups (later named genospecies; Ravin 1963) did frequently match phenotypically well defined species (taxospecies) gave paramount importance to hybridization results. Additionally, the occasionally found continua between phenotypically defined groups were usually resolved,

since organisms tended to be either closely related or not (Goodfellow et al. 1997). It is important to note here that DNA–DNA reassociation results are rough estimations of the average genetic relationship of two highly related organisms and that the actual sequence similarity of the compared DNA strands may be significantly higher. The interpretation of DNA–DNA hybridization results acquired predominance in the development of a species concept for prokaryotes; and their use over a period of decades has had an influence that cannot be underestimated. Nowadays, the idea of placing a group of organisms within a single group named 'species' is unavoidably linked to genomic coherency. However, there is a need to substitute such methods by others that give better scientific assistance (Stackebrandt et al. 2002), but such substitution in taxonomy could only be done if the new information retrieved confirms that of the standardized methods.

The genomic size of a species had been empirically circumscribed after the observation of how taxospecies fitted to genospecies. For some, cut-off values above 60% similarity (<7 °C of ΔT_m) would embrace coherent species (Johnson 1973). However, others might find more robustness by setting the boundaries as high as 80% similarity (<5 °C of ΔT_m; Grimont 1988). All such observations made an ad hoc committee recommend that a robust species definition could be circumscribed by the inclusion of organisms sharing more than 70% DNA similarity, or less than 5 °C ΔT_m (Wayne et al. 1987). However, such values were only a recommendation, since it had also been empirically observed that there was a transitional range of values (between 50 – 80% similarity, or 5–7 °C ΔT_m) where subgrouping could sometimes be complicated because different taxospecies could appear within a single genospecies and vice versa (Grimont 1988; Johnson 1989). Despite this, many scientists took the value of 70% as a rigid boundary for species circumscription, thereby unnecessarily forcing their descriptions (Rosselló-Mora 2003). Re-evaluations of the species definition have led to recommendations of more relaxed boundaries without rigid genomic boundaries for species circumscriptions but, in addition, the sound re-evaluation of such results, using additional taxonomic parameters (e.g. Stackebrandt et al. 2002; Ursing et al. 1995). It is clear that the original recommendations were produced after empirical observations were made with easily cultured organisms, such as enterobacteria (Grimont 1988; Stackebrandt 2003), anaerobic low-GC Gram-positive or Gram-negative organisms (Johnson 1973), or pseudomonads (Palleroni 2003). However, the use has undoubtedly been extended to a much wider range of organisms, as can be seen in the many new classifications. Given the vast diversity expected in the prokaryotic world (Whitman et al. 1998), it is clear that the parameters used to circumscribe the basic unit of diversity may not equally fit all organisms. Trying to evaluate the whole of microbial diversity with a single measuring stick is a reductionistic approach that

cannot be sound, especially if the parameters used in circumscriptions are taken as being rigid and immutable (Rosselló-Mora 2003).

The taxonomic schema should follow a pragmatic approach in order to provide the scientific community with an operative system (Rosselló-Mora and Kämpfer 2004; Young 2001). In this regard, it is accepted that the circumscription of the basic unit of prokaryotic classification should be based on the simultaneous evaluation of multiple parameters that cover both genomic properties and phenotype and that no single parameter is given undue prominence (Stackebrandt et al. 2002; Vandamme et al. 1996). DNA-DNA reassociation may not be regarded as the 'gold standard' for circumscribing species; but it has to be evaluated within the framework of a collection of parameters showing coherency in both genomic and phenotypic terms. For pragmatic reasons, it is recommended not to classify new species if one or either premise fails (Stackebrandt et al. 2002). For example, a clear-cut genomic group based on reassociation experiments that cannot be phenotypically distinguished from its related organisms may be regarded as a genomovar of a single species (Ursing et al. 1995). In a similar way, a clear-cut phenotypic group that cannot be genomically distinguished from its closest relatives should be considered as a biovar (Sneath 1992). Circumscription of a species within the framework of taxonomy must not simply rely on DNA-DNA reassociation results, although these are of paramount help to understand if one is dealing with a coherent group of strains that can be discriminated from their closest relatives.

Finally, there are some anecdotal examples where the relevance of DNA-DNA hybridization results has been disregarded when circumscribing prokaryotic species. Cases such as maintaining *Neisseria gohorreae* and *N. meningitidis* in two different species although genomically they should be one, or separating two genera such as *Shigella* and *Escherichia*, as well as many other examples for genera like *Yersinia*, *Bacillus*, *Brucella*, etc., respond to pragmatic reasons for their identification, often because of their medical implications. This was clearly stated by an ad hoc committee (Wayne et al. 1987) as: "phenotypic characteristics should agree with this definition and would be allowed to override the phylogenetic concept of species only in a few exceptional cases". This statement has also been ignored by many readers and such incongruities have been interpreted as unwarrantable pitfalls of the taxonomic principles (e.g. Palys et al. 1997; Sneath 1989; Stackebrandt 2003). It is worth emphasizing at this point that taxonomy pursues the construction of an operative, predictive and generally applicable classification schema. If the operationality of the system leads towards an impracticable but exhaustive classification, then the aim of taxonomy has failed. For pragmatic reasons, taxonomists are tolerant to the pitfalls of the measurements.

2.5 The Impact of DNA–DNA Hybridizations on the Conception of a Species and Changes in the Concept and/or the Definition

It is important to note here that the species is an artificial construct of the human mind basically addressed to classify the patterns of recurrence that can be observed in nature (Hey et al. 2003). The understanding of the prokaryotic world improved in parallel to technological developments, but some of these improvements have simultaneously fastened certain criteria in scientific belief, which over time have become tenets. One finds clear examples in prokaryotic taxonomy. The discrete units circumscribed by DNA–DNA reassociation which mostly agreed with a phenotypic framework were taken to represent those recurrence patterns understandable as species. That principle permitted the establishment of a rather stable and operative classification system for prokaryotes (Stackebrandt et al. 2002). However, there are criticisms of current circumscription because it is too conservative and because, by using the DNA–DNA reassociation circumscription criteria, no comparisons with higher eukaryote taxonomies can be carried out (see Rosselló-Mora and Amann 2001; Staley 2004). As is thoroughly discussed in eukaryotic taxonomy, the patterns of recurrence may be necessarily different for different kinds of organisms that exhibit distinct levels of morphological and/or physiological complexity (Hey 2001); and, thus, the parameters used to circumscribe species may be different for different taxonomies. Additionally, for given kinds of organism, one can view them from a variety of perspectives and, since each perspective is legitimate (Hull 1997), it is a question of accepting that pluralism in taxonomy may solve the so called 'species problem' (Ereshefsky 1998; Rosselló-Mora 2003; Young 2001). Taking such premises into account, a universal species concept may be impossible to achieve; and the basic essence of the prokaryotic species may not be comparable to any other species originating from other taxonomies. However, this is perhaps the most pragmatic position.

The principle of genomic coherency based on DNA–DNA reassociation results has had an influence on prokaryotic taxonomy comparable to that of 'breeding true' in the animal and plant species concept. The finding of a parameter that seems to unify criteria towards the recognition of recurrence patterns soon materializes as a tenet. For example, it is clear now that the 'breeding true' concept, which is the basis for the biological species concept (Mayr 1942), can no longer be taken as a universal parameter to embrace all eukaryotic species; and this has brought decades of heated debates (for reviews, see e. g. Hull 1997; Mayden 1997). Actually, the history of microbial taxonomy repeats that of eukaryotes and, in parallel to the

understanding of the extent of the organism's diversity, the validity of the circumscription parameters tends to be relative. Once, DNA–DNA reassociation was considered to be 'the gold standard' for many taxonomists for circumscribing species, such experiments were mostly used in the new descriptions and its use was even more reinforced after the recommendation of an ad hoc committee (Wayne et al. 1987). To date, taxonomists have succeeded in formulating a classification system of about 5,000 species, many of them circumscribed after DNA–DNA hybridization experiments were made available. Any change in the definition of the species should take that fact into account.

As has been discussed, the methods providing raw genomic similarities are submitted to a relatively large experimental error, in addition to impractical properties such as the impossible construction of an interactive and cumulative database (Sneath 1989; Stackebrandt 2003). These are indeed important pitfalls of the method that can lead to its use towards the emerging technologies being questioned (Stackebrandt et al. 2002). Actually, an ad hoc committee for the evaluation of the current definition of species (Stackebrandt et al. 2002) has recommended the search for new methods to replace the use of DNA–DNA reassociation experiments. Special emphasis is being placed on the evaluation of methods such as: (a) sequencing protein-coding genes, an extension of MLST, or (b) DNA profiling, such as AFLP, ribotyping, REP-PCR, or PCR-RFLP. However, any method that is to be used as a substitute for DNA–DNA reassociation should be previously validated. The reluctance to use sets of genes for their phylogenetic evaluation is mainly due to the difficulties in selecting them and designing proper amplification primers and, as criticized for the 16S rDNA analysis, also corresponds to the insignificant portion of the genome that they represent. Indeed, there have been some attempts to design universal primers for some of the reduced sets of universally present genes, but only with about 60% amplification success (Santos and Ochman 2004). Primer redesign or improvement can only be carried out if the genome of closely related organisms is available. However, this approach becomes very impractical when the new isolates belong to unknown phyla. Yet, it seems that there might be a correlation between some single gene sequence identities and genomic similarities (Zeigler 2003), especially the *recN* and *dnaX* genes that have been selected as being discriminative between species. However, as the author also claims, it is too soon to place strong emphasis on this because the data set used was very limited, and all genomes analysed belonged to pathogenic or human saprophytic microbiota.

In principle, reassociation experiments represent raw data on whole genome comparisons, which is an advantage for those techniques that analyse a reduced portion of the genome (Mallet and Willmott 2003; Young 1998). Of course, the best substitute for reassociation experiments in tax-

onomy would be pure genome comparisons after undertaking complete sequencing programs, but this is still utopian because of the relatively high costs of sequencing. Despite the technical difficulties in achieving complete genomes, the first insights into their comparisons and the concordances with classic taxonomic circumscriptions are ongoing, and encouraging (Zeigler 2003). For example, Konstantinidis and Tiedje (2005) carried out an exhaustive comparative survey of about 70 closely related and completely sequenced genomes and their corresponding hybridization values. The best parameter found for taxonomic purposes was the average nucleotide identity (ANI) of shared genes. The values obtained correlated with both 16S rRNA gene sequence identity and DNA–DNA similarity values with pairwise comparisons. Nevertheless, it is still too soon to be able to use this parameter, since there are many comparisons still to carry out before it can be validated. However, the final goal of such techniques in taxonomy should be to undertake the comparisons using the understanding of the information behind the genes or genomes that are under study. Ignoring this fact and treating genes or genome information as mere quantitative data would mean that the substitution would not result in an improvement to the use of DNA–DNA reassociation experiments.

2.6 Epilogue

The species concept for prokaryotes has been especially devised by taxonomists to create an operative and predictive classification system. The first formulation of what a species could be was made by Aristotle about 2,400 years ago and the idea of species was understood as being the basis for a hierarchic classification schema. Since then, the concept of 'species' should be regarded as a property of taxonomy; and its formulation has been improved by taxonomists in parallel to conceptual and methodological scientific developments. Other uses of the term to name essentially different units has led to heated debates, but as Sneath (1988) remarked, taxonomy has been the primary basis for conceptual developments in evolution (and I would say also ecology). The species concept for prokaryotes is well consolidated in microbial taxonomy, but of course it can be improved. DNA–DNA reassociation results gave, for the first time, a measurable way to circumscribe units and therefore the use of the method was established as a priority when classifying new species. This gave the concept a 'genomic coherency' dimension that cannot be misinterpreted and which may equally apply to the 'phenotypic coherency' and 'monophyly' dimensions provided by established taxonomic approaches. Consequently, I am reasonably confident that most taxono-

mists would agree that it is the best concept that we can achieve at this point in time.

DNA–DNA reassociation experiments applied to taxonomy should be taken as a method that allows raw genomic coherency to be understood. This means that, when analysing a group of strains that appear monophyletic and are genetically and phenotypically related, the hybridization results will help to show if they belong to the same genomic circumscription or not. Rigid boundaries, such as 70%, are not to be taken dogmatically, but one has to understand that the classification of new species should follow pragmatic and logical premises. In some cases, a defined phenotypic and genetic group will be circumscribed by cut-off values of 60% or even 50%, but they could still be considered as a single species. In other cases, if the phenotypic and genetic information supports them, two different species may even be distinguished by cut-off values of 80%. The most important point here is that when describing new species, no single value can be given undue prominence, and, altogether, the information retrieved should show enough consistency for the classification. Classifying new species when they cannot be differentiated from their closest relatives hinders the operationality of the system. The aim of a taxonomist is not the classification of everything as a means to an end, but to provide a system that can be used by the rest of the scientific community who find it easy, useful and workable.

DNA–DNA reassociation experiments have been predominantly taken as the measuring basis for circumscribing species for nearly half a century. Most of the current taxonomic schema have been constructed with them and they have been of paramount importance in the way we understand prokaryotic classification. Nevertheless, such techniques suffer from important disadvantages, especially when compared with the newly emerging molecular approaches. Sooner or later, DNA–DNA reassociation will be replaced by analyses that provide more accurate measurements and cumulative databases. However, given the influence that genomic similarities have had on the circumscription of most of the species during the construction of the current classification schema, new methodologies may have to reproduce similar observations. Whole genome sequence comparisons are surely the choice for replacement, and parameters such as ANI could be of enormous help in understanding genomic coherency. This will be true though only if these new species definitions render units that are comparable to the hitherto classified species and that represent the basic structure of our current, indeed defective, but operative and predictive taxonomic classification system for prokaryotes. However, for the time being and until whole genome sequencing is as routine as single gene sequencing is now, DNA–DNA reassociation experiments will have to be used to circumscribe species.

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